Molecular Characterization of Human Regulatory T Cells in Health and Disease

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Wo ein Wille ist, ist auch ein Weg.
Where there's a will, there's also a way.

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Contents

Abbreviations

APC	antigen-presenting cell
CV	coefficient of variance
CCR	chemokine (C-C motif) receptor
ChIP	chromatin immunoprecipitation
CD	Crohn's disease
CTLA4	cytotoxic T lymphocyte associated protein 4
DC	dendritic cell
FC	fold change
FDR	false discovery rate
FACS	fluorescence activated cell sorting
FOXP3	forkhead box protein 3
GvHD	graft-*versus*-host disease
IBD	inflammatory bowel disease
ICAM	intracellular adhesion molecule
IL	interleukin
LGALS3	galectin 3
LP	lamina propria
MACS	magnetic cell sorting
MHC	major histocompatibility complex
NK	natural killer cell
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
RCC	renal cell carcinoma
RPS9	ribosomal protein S9
RT	reverse transcription
SAM	significance analysis for microarrays
TCR	T cell receptor
$T_H1,2,3$	T helper 1,2,3 cell
T_R1	T regulatory 1 cell
T_{Reg}	regulatory T cell
TREC	T cell receptor excision circle
TNF	tumor necrosis factor
TNFRSF	TNF receptor super family
TRAF1	TNF-receptor associated factor 1
UC	ulcerative colitis
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

1 Introduction

1.1 Principles of microarray technology

Microarrays are one of the fundamental tools for research in life sciences. The term "microarray" refers to its characteristic feature to arrange (or "array") a high number of molecules on a very small ("micro") area. Despite the variety of technical solutions for performing microarray analysis, all are miniaturized hybridization assays, enabling studies of thousands of targets simultaneously. All microarray experiments share the following key components:

- the array itself, containing the immobilized "targets" arranged in a regular pattern,
- the labelled sample or "probe" that is hybridized to the microarray and
- a detection system that quantifies the hybridization signal [1, 2].

1.1.1 Microarrays

Microarrays consist of a collection of target molecules immobilized onto a solid support so that they form tiny features, called "spots". Spot size varies among systems, but is usually less than two hundred micrometers in diameter. A glass slide or silicon wafer acts as the solid support providing reactive groups (e.g. aldehyde, amine, epoxy or ester modifications) enabling immobilization of the target molecules to the surface. Microarrays can be classified according to the nature of the spotted targets into DNA-, protein- and tissue chips. DNA microarrays are further divided into cDNA and oligonucleotide microarrays referring to immobilization of either double-stranded PCR products or shorter single-stranded synthesized oligos. For DNA microarrays more than one million spots can be arrayed in a total area of a few square centimeters (Affymetrix). Two parallel approaches have been developed for the production of microarray slides. Nucleic acid targets can either be synthesized directly onto the microarray, or purified target molecules can be deposited in a contact-dependent or independent printing method using microarray spotter equipment. This latter approach is increasingly advantageous allowing easy spotting of customized microarrays, while *in-situ* synthesis (as pioneered by Affymetrix) is characteristic for commercially available formats due to higher efforts but also increased purity [1, 2].

1.1.2 Probe labelling

The microarray sample that is supposed to be analysed, e.g. mRNA for gene expression studies or DNA derived from genomic analysis, is converted to a labelled population of several thousands of nucleic acid molecules, the probe. Incorporation of tagged nucleotides can be achieved during cDNA-synthesis as well as during an *in vitro* transcription reaction yielding cRNA probes. Thereby, hybridization of cRNA samples to DNA microarrays can be carried out at higher temperatures and thus increased specificity, because RNA-DNA-hybridizations are energetically advantageous. Furthermore,

cRNA synthesis provides the possibility for linear amplification of the original starting material, allowing performance of microarray experiments even for samples of very small cell numbers. However, a disadvantage of the use of RNA as a probe is its fragility, demanding especially careful sample preparation to prevent RNA degradation. Fluorescent dyes, like the cyanine dyes Cy3 and Cy5, have been established as the predominant label in microarray experiments and have almost completely replaced radioactive methods. For direct labelling procedures, fluorescence conjugated nucleotides, such as Cy5- or Cy3-UTP, are incorporated during cDNA or cRNA synthesis, while indirect strategies are based on biotin- or aminoallyl-tagged nucleotides. The indirect approach allows higher labelling efficiency due to reduced steric hindrance because the incorporated nucleotides possess a spacially smaller modification. Additionally, it is the more robust method, as labelling is only carried out after hybridization, thereby preventing premature photobleaching of the used fluorescence dye during sample handling in the light. Recently, two-channel or competitive hybridization approaches are gaining more importance. Therefore, the control sample and the probe-of-interest are labelled with different fluorescence dyes, enabling comparative analysis of both samples on the same microarray thereby reducing technical variance [1, 2].

1.1.3 Microarray hybridization

For microarray hybridization, the labelled fragments in the probe are expected to form duplexes with their immobilized complementary targets. This requires that the nucleic acids are single-stranded and complementary as well as accessible to each other. The number of duplexes formed reflects the relative number of each specific fragment in the probe, as long as the amount of immobilized target is in excess and not limiting hybridization kinetics. By measuring the fluorescence signals associated with each spot, the relative abundance of specific sequences in the sample can be determined [1, 2].

1.1.4 Scanning and data analysis

Microarray scanner typically contain two different lasers that emit light at wavelengths suitable to excite the fluorescent dyes used as labels. A confocal microscope attached to a detector system records the emitted light from each of the microarray spots, allowing high-resolution detection of the hybridization signals. Despite their small size, microarrays generate large quantities of data even from a single experiment. As a typical experiment will involve analysis of several samples on replicate arrays, the use of computerized data processing is necessary to handle the huge amount of data created and to gain maximum information. This can be achieved by specialized software that calculates raw signal intensities from scanned microarray slide images and normalizes this data to remove the influence of experimental variation. Application of statistical tools facilitates extraction of significant data and increases reliability of drawn biological conclusions. Additionally, visualization software can be used for further data mining

providing helpful features, such as data integration and pattern recognition. Clustering data points can be used to identify co-regulated genes which possibly belong to the same pathway [1, 2].

1.1.4.1 SAM - Significance analysis of microarrays As DNA microarrays enable expression measurement of thousands of genes in a single hybridization experiment, massive amounts of data are generated. That is why methods are needed to determine whether changes in gene expression are experimentally significant. Clustering analysis of microarray data can find coherent patterns of gene expression [3] but provides only little information about statistical significance. Methods based on conventional t tests calculate the probability (P) that a difference in gene expression occured by chance. But while $P = 0.01$ is significant for evaluation of experiments involving small numbers of genes, already 100 false positive genes would be identified in a microarray experiment including 10,000 genes [4]. This problem led to the development of Significance Analysis of Microarrays (SAM), a statistical method adapted specifically for microarrays [5]. SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific t tests. To each gene a score is assigned on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR). Genes with scores greater than an adjustable threshold are deemed potentially significant.

1.1.4.2 Hierarchical clustering Transcriptional profiling techniques promise a wealth of data that can be used to develop a more complete understanding of gene function, regulation and interactions. The simplest way to find interesting candidate genes is to search for those that are consistently up- or down-regulated by running a statistical data mining tool such as SAM (see chapter 1.1.4.1). But identifying common patterns of gene expression and grouping genes into expression classes might provide much greater insight into their biological function and relevance. Based on our understanding of cellular processes, genes that are contained in a particular pathway, or that respond to a particular environmental challenge, should be co-regulated and consequently, show similar patterns of expression. To identify those genes with similar expression patterns, a large group of statistical methods exists, generally referred to as "cluster analysis". First, an "expression vector" for each gene is defined representing its location in n-dimensional "expression space", where n is the number of experiments. Afterwards, distances between all objects are calculated so that clustering algorithms can sort the data and group similar genes together. In parallel, it is also possible to apply the clustering algorithm to analyse and group experiments. Various clustering techniques exist differing in distance similarity measurement and the data sorting algorithm. Most of them are hierarchical where the generated classification has an increasing number of nested classes and the result resembles a phylogenetic tree.

To facilitate interpretation of the results of a clustering analysis, an intuitive visual representation is helpful. One approach is to create an expression matrix in which each column represents a single experiment and each row represents the expression vector for a particular gene. Each matrix element is coloured on the basis of its expression value thereby creating a visual representation of gene expression patterns across experiments. The most commonly used method is to colour up-regulated genes in red, down-regulated genes in green and genes without change in expression in black - with their relative intensity representing their relative expression [6].

1.1.4.3 From genes to pathways Identifying hundreds of genes whose expression is markedly different in one sample than in another provides a large amount of potentially valuable data. The trick is to focus on the genes that are relevant to the questions being asked. One way to do so is to find a subset of genes that are functionally connected through common pathways [7]. Recently, software for metabolic pathway analysis supports examination of gene expression profile data by enabling determination of direct binding and interacting molecules.

1.1.5 Applications of microarray analysis

The versatility of microarray analysis is confirmed by its rapid development as a general technique in molecular biology. Microarrays have not become a replacement to established procedures but a novel, high-throughput approach. Enabling simultaneous measurement of thousands of targets in a single assay, microarrays have emerged as an alternative for previously time-consuming analyses. They offer almost unlimited applications and are thus employed for gene expression and genome analysis as well as for drug discovery, analysis of protein-protein- or DNA-protein-interactions, identification of specific antibodies and promoter regions. Completion of the human genome project, together with emerging information about genomes of many pathogens, additionally offers new possibilities for exploring the molecular pathogenesis of infectious diseases. Alone in this field, chip technology can be applied to a wide variety of scientific questions, such as interaction studies between microorganisms and the host, pathogen studies to identify known or novel organisms as well as investigations of pathogenic determinants [8]. In biomedical research, microarrays have become essential tools for characterizing transcriptomes of different cell types in diverse diseases, such as inflammatory bowel disease [9], renal cell carcinoma [10, 11] and severe aplastic anemia [12] or to study e.g. immune responses as a whole [13].

1.1.5.1 Gene expression analysis Gene expression analysis examines the composition of cellular mRNA populations. Identity and expression levels of transcripts constituting the cell's transcriptome provide insights into cell state and gene activity. As the precursors of translated proteins, changes in mRNA levels can be linked to changes in the proteome. A typical microarray gene expression experiment compares relative

expression levels of specific transcripts in two probes, one control and another sample, derived from cells whose response or status is being investigated [1, 14]. These studies examine e.g. the effects of treating cells or patients with certain substances (chemicals, pharmaceuticals, infection with pathogens, [12]), the consequences of over-expression of genes-of-interest in transfected cells [10, 15, 16], and mutant *versus* parental strains to delineate functional pathways [17, 18]. Furthermore, time course experiments can help to reveal molecular processes during development and differentiation of diverse cell types [19]. In cancer research, microarrays are used to reveal differentially expressed genes in transformed cells and metastases, to identify diagnostic markers, and to classify tumors based on their characteristic gene expression profiles [20, 21]. The obtained data are often correlated to prognosis and/or response to therapy [11]. Not only in cancer, but in human diseases in general, gene expression profiling studies have allowed better understanding of pathophysiological processes. They may lead to the developement of new clinical tools to improve diagnosis and prognosis of patients [9, 12].

1.1.5.2 Genomic analysis Microarrays are useful tools for genomic analyses. Identification of new genes by examining nucleic acid sequences derived from open reading frames has proven to be an efficient approach of annotating the human genome [1, 2]. Elucidation of interactions between transcription factors and their target genes can advance understanding of gene regulation. That is why ChIP-chip-technology combines immunoprecipitation of transcription factor-DNA complexes and genomic microarray-based hybridization of these DNA fragments [22]. Furthermore, microarrays can be applied for prediction of splice variants of transcripts and are recently used for genotyping of single nucleotide polymorphisms (SNP) reflecting DNA sequence variations among individuals [23]. Samples can be sequenced by microarray hybridization, thus providing convenient means for identifying new genetic variants. Correlation of expression data and SNP genotyping in so called linkage analysis may reveal new susceptibility genes of diverse diseases [24, 25].

1.1.5.3 Drug discovery As a typical drug discovery process takes several years, incurs high costs and turns only a few candidates into approved drugs, methods for increasing efficiency of this procedure are highly welcomed. Microarrays can provide useful information in the different stages of drug discovery. Identification of potential drug targets can be supported by elucidating metabolic pathways when looking at coexpressed genes. Once drug targets have been selected, microarrays can be used to define their toxicity by examining expression profiles induced by drug treatments. Additionally, different function modes of drugs can be revealed based on the gene expression changes they elicit [1, 2, 26].

1.1.6 Customized *versus* commercial microarrays

The amount of both biological and technical replicates required for reliable biological results increases the number of arrays needed for clinical trials or disease profiling and screening. Utilization of commercial platforms usually targeted at global screening of gene expression is relatively expensive, especially when multiple samples should be analysed. Customized microarrays offering a targeted selection of genes-of-interest can be an affordable method-of-choice for focussed studies, often following genome-wide expression profiling [13]. That is why apart from commercially available formats (e. g. Affymetrix, CodeLink...), diverse customized microarrays have recently captured the market. Such specialized microarray approaches have been described for different purposes enabling mostly cancer [11, 27], disease [9] or immunological studies [13, 28].

1.2 The immune system

The immune system is a society of interacting cells consisting of T and B lymphocytes, natural killer (NK) cells, macrophages, professional antigen-presenting cells (APCs) and their various subclasses. Macrophages and dendritic cells are part of the innate immune system, while the lymphocytes constitute the arm of adaptive immunity. B lymphocytes, NK cells and macrophages mature in the bone marrow and in the fetal liver, while T cells develop in the bone marrow and in the thymus. T and B cell development share many features, yet differ in others [29]. Cells of the immune system work together as a team to protect the host against foreign pathogens. After an attack by infectious agents, professional APCs, dendritic cells (DCs) in particular, present antigenic peptides of the intruders to T cells. Antigen presenting major histocompatibility complexes (MHC), costimulating cell-surface molecules on APCs and cytokines drive T cells into clonal expansion. Then these T cells in turn communicate with other T or B cells to regulate their responses. After a peak phase with the highest clonal expansion of reactive cells, the immune response undergoes a down phase. Most lymphocytes are eliminated, while the few that survive constitute the pool of memory cells [30].

1.2.1 B lymphocytes

B cells express cell membrane receptors (antibodies) with a single antigen specificity. Millions of different B cells produce antibodies that can potentially capture millions of antigens thereby making up the so-called antibody repertoire. B cells are selected in the bone marrow on the basis of the affinity of their antibodies: cells equipped with B cell receptors of high affinity for proteins derived from self tissues are eliminated. Mature B lymphocytes leave the bone marrow and populate the secondary lymphoid organs - spleen and lymph nodes as well as the gut-associated lymphoid tissues. Once activated by an antigen, B cells undergo a second round of selection in the follicles of secondary lymphoid organs, mature into plasma cells producing and secreting antigen-specific antibodies and then recirculate to the bone marrow [30]. These secreted antibodies are the main effectors of the humoral immunity as they bind and thereby neutralize their specific extracellular antigen [29].

1.2.2 T lymphocytes

Pre-T lymphocytes emigrate from the bone marrow into the thymus. There, they mature and are positively or negatively selected, depending on the affinity of their T cell receptors (TCRs) for self antigens presented by MHC. MHC class I and II are molecules that sample peptide fragments from foreign and self proteins, respectively. They display these peptides at the cell's surface for scrutiny by T cells in a process known as antigen presentation. Each MHC class I or II protein presents a unique fragment and thousands of MHC molecules protrude from every cell. Most of the peptides presented in the thymus are derived from self proteins. T cells with a high

affinity for self MHC molecules and peptides are eliminated to ensure tolerance to normal tissue and to prevent autoimmunity. T cells that interact with MHC class I molecules develop into $CD8^+$ T lymphocytes, while those with affinity for MHC class II proteins turn into $CD4^+$ T cells. Only mature T cells with a functional TCR leave the thymus and populate the secondary lymphoid organs. Mature $CD4^+$ T cells function as T helper (T_H) cells and secrete cytokines that regulate either cellular immune responses by activating macrophages (T_H 1 or inflammatory T cells) or antibody responses by driving differentiation of naive B cells into plasma cells (T_H 2 cells). Activated $CD8^+$ T lymphocytes serve as cytotoxic effector cells by eliminating cells infected with viruses or bacteria [29, 30].

1.2.2.1 T cell activation The first step in adaptive immunity is the activation of naive antigen-specific T cells by APCs. This process takes place in the lymphoid tissues and organs through which naive T cells are continuously passing. The most important feature of APCs is the expression of co-stimulatory molecules, of which B7.1 and B7.2 are the best characterized. Naive T cells will respond to antigen only, when an APC presents both a specific antigen (complexed with MHC) to the TCR and a B7 molecule to CD28, the corresponding receptor on the T cell. Three cell types can serve as APCs: DCs, macrophages and B cells, each possessing a distinct function in eliciting immune responses. Tissue DCs take up, process and present antigens and are stimulated by infection to migrate to local lymphoid tissues. There they differentiate into mature DCs expressing co-stimulatory activity. They are the most important activators of naive T cells *in vivo*. Macrophages efficiently ingest particulate antigens such as bacteria and are induced by infectious agents to express MHC class II and co-stimulatory molecules. The unique ability of B cells to bind and internalize soluble protein antigens *via* their receptors may be important in activating T cells to this class of antigen, provided that co-stimulatory molecules are also induced on the B cell. In all three cell types of APC, the expression of co-stimulatory molecules is activated in response to signals from receptors that also function in innate immunity to signal the presence of infectious agents. The activation of naive T cells by APCs leads to their proliferation and differentiation into armed effector T cells. This process depends on the production of cytokines, in particular the T cell growth factor interleukin-2 (IL2), which binds to a high affinity receptor on the activated T cell. T cells, whose antigen receptors are ligated in the absence of co-stimulatory signals, fail to produce IL2 and instead become anergic or die. This dual requirement for both TCR ligation and co-stimulation by the same APC helps to prevent naive T cells from responding to self-antigens on tissue cells, which lack co-stimulatory activity. Sufficiently activated T cells develop into armed effectors, the critical event in most adaptive immune responses. Once an expanded clone of T cells achieves effector function, its progeny can act on any target cell that displays antigen on its surface - without further need of co-stimulatory signals. Thereby, effector T cells have a wide variety of functions: CD8 cytotoxic T cells

kill infected cells, while T_H1 cells activate macrophages, and both together make up cell-mediated immunity. T_H1 and T_H2 cells are stimulating B cells to produce different classes of antibodies, thus driving humoral immune response [29].

1.2.2.2 Central *versus* peripheral tolerance One of the probably most striking capacities of the immune system is its ability to discriminate between self and non-self, thereby preventing autoimmune responses while allowing effective immunity against infections. Several mechanisms have evolved for the maintenance of tolerance and immune homeostasis [Figure 1]: Self-reactive T cells are deleted during their development in the thymus in a process known as central tolerance. Thereby, most T cells are eliminated by positive or negative selection, sparing only those T cells equipped with receptors exhibiting a moderate affinity to foreign antigenic peptides presented by self MHC molecules. All T cells that do not express a TCR or a TCR that fails to recognize self MHC complexes undergo apoptosis. Furthermore, T cells carrying a TCR with a high affinity to self MHC complexes are also deleted by programmed cell death.

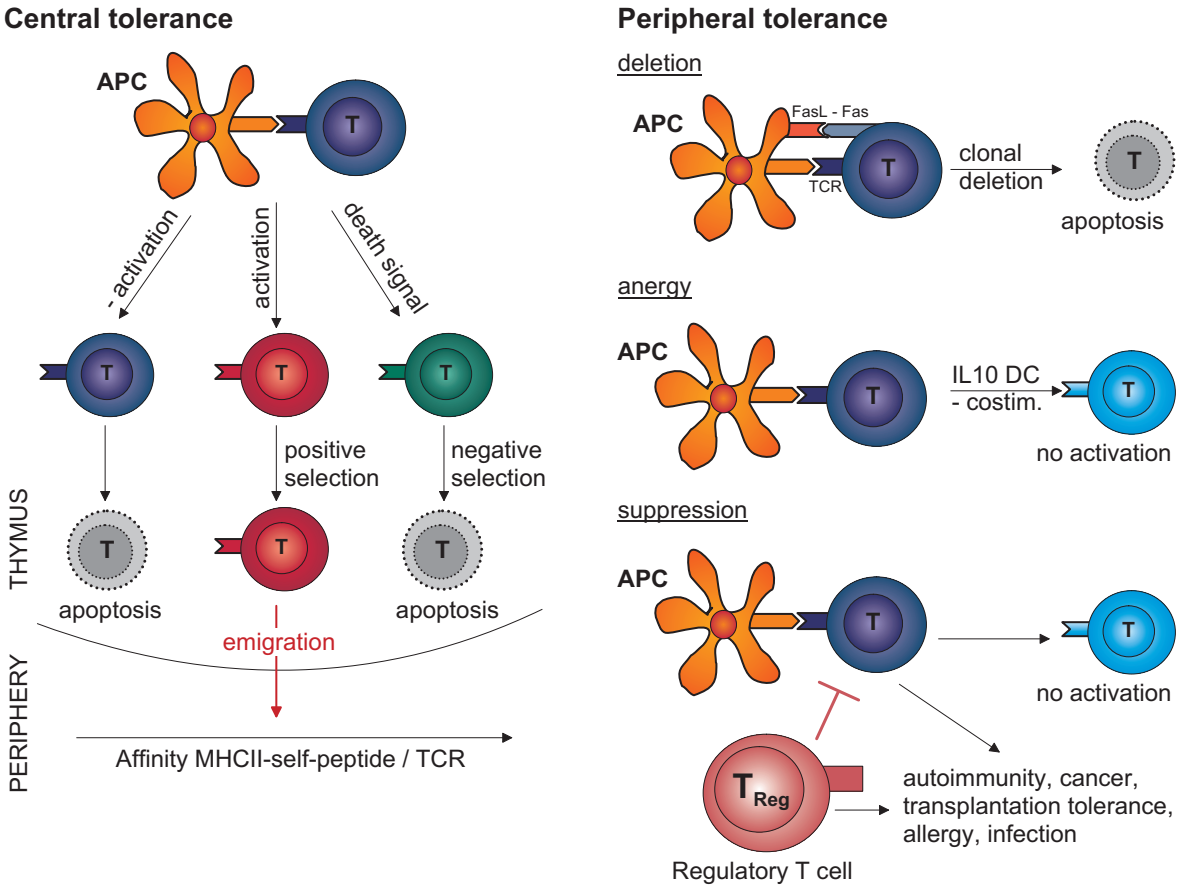

Figure 1: Mechanisms of central and peripheral tolerance [34]

But as this negative selection is incomplete, self-reactive T cells that have escaped from clonal deletion, need to be controlled in the periphery. Peripheral tolerance mechanisms to prevent activation of autoreactive T cells include deletion, anergy and

suppression [Figure 1]. The so-called regulatory T cells (T_{Reg}) actively suppress activation and expansion of these self-reactive escapees [31, 32, 33, 34]. In this way, T_{Reg} cells control the delicate balance between immunity and tolerance [35], explaining their important role in autoimmune diseases [36, 37, 38], cancer [39, 40, 41], transplantation tolerance [42, 43, 44] and even allergy [45, 46] as well as for the outcome of infections [47].

1.2.2.3 Regulatory T cell populations As summarized in Figure 2, several types of T_{Reg} cells have been described in both mice and humans, including naturally occurring or professional ($CD4^+CD25^+$) and adaptive or inducible (T_H3 and T_R1) T_{Reg} cells.

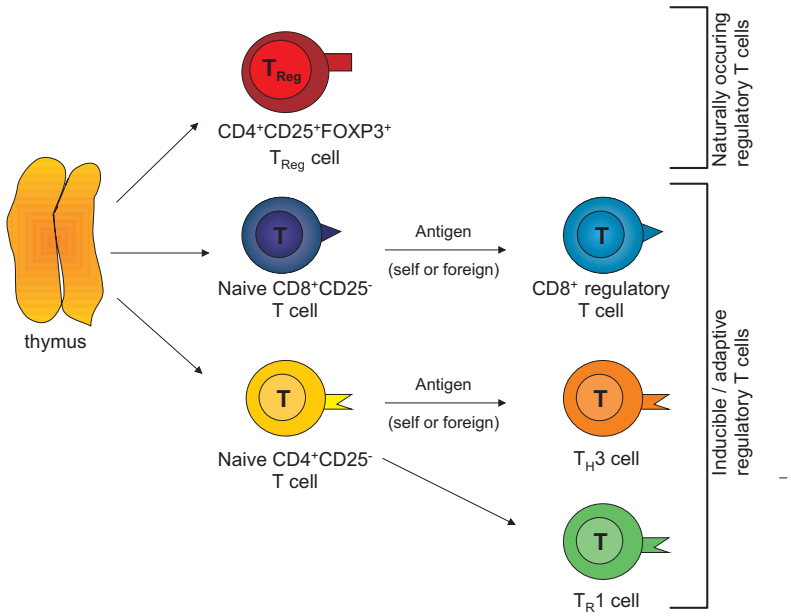

Figure 2: Naturally occurring and inducible regulatory T cells [34]

Naturally occurring T_{Reg} cells constitutively express the cell surface molecule CD25 [48] and the transcriptional repressor FOXP3, an essential key gene controlling their development and function [49, 50, 51]. These cells mature and migrate directly from the thymus and constitute approximately 2-4% of total human $CD4^+$ T cells [52, 53]. They represent an endogeneous long-lived population of self-antigen-specific T cells in the periphery. As a potential consequence of their positive selection in the thymus naturally occurring T_{Reg} cells are rendered anergic and become able to produce anti-apoptotic molecules which protect them from negative selection [54]. In humans, T_{Reg} cell activity is restricted to the $CD4^+CD25^{high}$ population, while $CD4^+CD25^{low}$ T cells represent mainly previously activated T helper cells and naive T cells reside within the $CD4^+CD25^-$ population [55, 56]. Other populations of T_{Reg} cells can be induced from naïve $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells in the periphery under particular conditions of suboptimal antigen exposure and/or co-stimulation, such as the influence of semi-mature dendritic cells, interleukin-10 (IL10), transforming growth factor-β (TGF-β)

and possibly interferon-α (IFN-α) [57, 58]. Distinct subtypes of these adaptive T_{Reg} subpopulations are included in the $CD4^+$ T cell compartment and are distinguished as T regulatory 1 (T_R1) and T helper 3 (T_H3) cells according to their different cytokine profiles [59, 60]. T_R1 cells secrete high levels of IL10, no IL4 and no or low levels of IFN-γ, while T_H3 cells secrete high levels of TGF-β. Although $CD8^+$ T cells are normally associated with cytotoxic T-lymphocyte function and IFN-γ production, a subtype of these cells has been described to secrete IL10 and to possess regulatory capacities [61, 62]. Just to complete the picture, NK and B cells can also display regulatory activity [29]. All these different T cell subpopulations with regulatory function coexist and contribute to immunosuppression [63].

1.2.2.4 Marker molecules of regulatory T cells Isolation, definition and characterization of regulatory T cells remain difficult as the availability of specific marker molecules is still limited [35]. Apart from CD25, additional surface molecules have been reported as associated with T_{Reg} cell function [Figure 3], e.g. cytotoxic T-lymphocyte-associated protein 4 (CTLA4) [64, 65, 66], tumor necrosis factor receptor superfamily, member 18 (TNFRSF18 or GITR) [67, 68] and selectin L (SELL or CD62L) [69, 70]. Furthermore, FACS analyses revealed that T_{Reg} cells express high surface levels of HLA-DR, IL2 receptor γ-chain (IL2RG, CD122) and CD45RO [47].

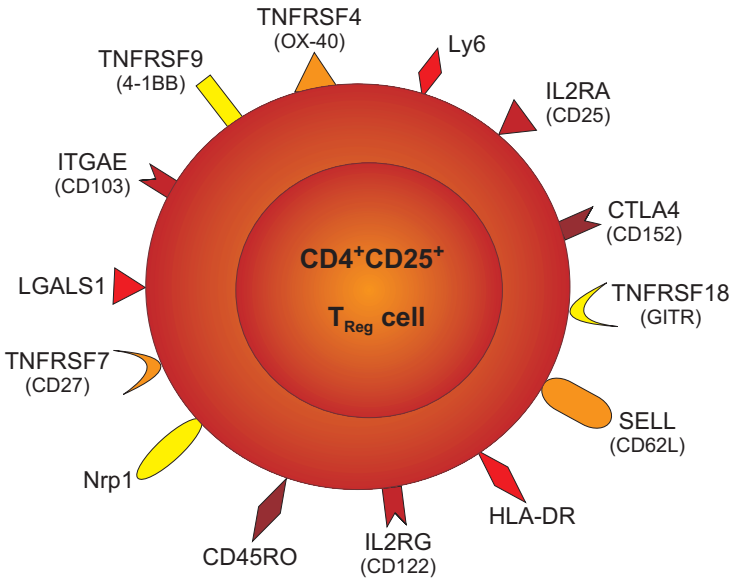

Figure 3: Surface molecules on $CD4^+CD25^+$ T_{Reg} cells in mice and men. Studies have reported several different molecules preferentially expressed on the surface of naturally occurring $CD4^+CD25^+$ T_{Reg} cells in mice and men. However, the vast majority do not remain as faithful biomarkers after T cell activation and do not appear to be convincingly involved in the mechanisms underlying T cell suppression (modified according to [32]).

IL2RA = interleukin-2 receptor alpha, ITGAE = integrin alpha e, LGALS1 = galectin-1, IL2RG = interleukin-2 receptor gamma, TNFRSF = tumor necrosis factor receptor superfamily, Nrp1 = Neuropilin-1, CTLA4 = cytotoxic T-lymphocyte-associated protein 4, SELL = selectin L

However, all these molecules are also expressed by naive $CD4^+CD25^-$ T cells upon activation, thereby hampering discrimination between regulatory and conventionally activated $CD4^+$ T cells [31]. Furthermore, CD25 as well as other T_{Reg} cell molecules, such as GITR and CTLA-4, are not expressed on all $CD4^+$ T cells with regulatory function [71]. Recently, new genes like Neuropilin-1 (Nrp1) for mouse [15] and CD27 coexpression with CD25 for human [72] were suggested as useful markers to distinguish regulatory from effector T cells. Like murine cells, human $CD4^+CD25^+$ T_{Reg} cells express significantly more FOXP3 (forkhead box P3) mRNA and protein than $CD4^+CD25^-$ T cells do. But, in contrast to data obtained from mouse models, overexpression of FOXP3 in human $CD4^+CD25^-$ T cells alone is not sufficient to generate potent suppressor T cells *in vitro*, suggesting additional factors required for the development, differentiation and function of human T_{Reg} cells [16]. Recent reports in humans even demonstrated induction of FOXP3 gene and protein expression in activated conventional T cells without suppressive activity [16, 73]. Furthermore, the usefulness of FOXP3 as a marker of T_{Reg} cells is limited by its nuclear localization, precluding staining of living cells [74]. However, human $CD4^+$ T cells expressing CD25 represent a heterogenous cell population containing not only regulatory but also recently activated effector/memory T cells [75]. Peripheral $CD4^+CD25^+$ T_{Reg} cells in healthy adults display a memory phenotype ($CD45RA^-$ $CD45RO^+$and $CD45RB^{low}$) indicating previous experience with their antigen *in vivo* [76].

1.2.2.5 Characteristics and suppression by T_{Reg} cells Despite recent advances in T_{Reg} cell biology, the mechanism of immunosuppression by human $CD4^+CD25^+$ T_{Reg} cells remains elusive. It has been shown that naturally occurring $CD4^+CD25^+$ T_{Reg} cells are characterized by an anergic phenotype, because they do not proliferate upon TCR stimulation *in vitro*. They exist without specific antigen stimulation and actively suppress immune responses in a cell-cell contact-dependent manner [77]. This mechanism is related to the combined action of surface CTLA4 and TGF-β leading to the inhibition of the IL2 receptor alpha chain on target T cells [54]. In contrast to that, antigen-induced T_{Reg} cells exert their immunosuppressive function through the secretion of soluble factors, such as IL10 or TGF-β, which are both cytokines that can inhibit T cell proliferation [77]. Furthermore, both cytokines can also act on APCs and DCs. IL10 e.g., can affect differentiation of DCs by preventing the secretion of IL12 and thus impairing the ability of DCs to promote T cell activation and T_H1 differentiation [29]. To suppress activation, proliferation and IL2 production of $CD4^+$ as well as $CD8^+$ T cells, both mouse and human T_{Reg} cells need to be activated *via* their TCR [47]. Antigen specific as well as polyclonal TCR stimulation can induce T_{Reg} cells to show active proliferation *in vivo*. Their suppressive capacity is most likely antigen non-specific, i.e. they are able to suppress not only proliferation of T cells with the same antigen specificity but also those directed against irrelevant antigens [76]. Recent *in vitro* studies suggest that there might be both a primary contact-dependent and a

secondary secretion-dependent suppression, which seem to be antigen-nonspecific. The primary suppressive mechanism is thereby mediated through cell-cell-contact by T_{Reg} cells and $CD4^+CD25^-$ T cells inducing anergy due to cell cycle arrest in the latter subset. Secondary suppression is based on the cytokines IL10 and/or TGF-β secreted by the anergized $CD4^+CD25^-$ T cells, that in turn suppress proliferation and activation of other $CD4^+CD25^-$ T cells. This model illustrates the phenomenon of "infectious tolerance". Given the rather low proportion of T_{Reg} cells and their dependency on cell contact to mediate suppression, infectious tolerance might help to explain how T_{Reg} cells can regulate immune homeostasis through this bystander mechanism *in vivo* [78]. Another interesting aspect is how T_{Reg} cells themselves are regulated. To date it is speculated, that activation of T_{Reg} cell mediated suppression depends on the strength of TCR stimulation, co-stimulatory signals and cytokine repertoire. Thereby, self as well as non-self antigens are capable to induce T_{Reg} cell activation [47]. However, it seems likely that T_{Reg} cells possess multiple mechanisms of mediating suppression - depending on the particular biological situation.

1.2.2.6 T_{Reg} cells in autoimmunity Autoimmune diseases afflict approximately 5% of the population and reflect a failure in the immune system to discriminate between self and non-self resulting in the breakdown of self-tolerance. T_{Reg} cells have been shown to play an important role in the maintenance of immune homeostasis and self-tolerance by counteracting the development and effector functions of potentially autoreactive T cells. Thus, an imbalance of natural T_{Reg} cells and self-reactive T cells towards a dominance of the latter seems to be responsible for the development of autoimmunity [79, 245].

1.2.2.6.1 Model systems *In vivo* studies on the role of T_{Reg} cells in autoimmune diseases have been carried out in different murine model systems. *Nude* (*nu/nu*) mice are T cell deficient due to a mutation in the gene for the transcription factor Whn, which makes them incapable of developing a thymic epithelium. Injection of syngeneic peripheral $CD4^+CD25^-$ T cells into *nude* mice leads to induction of organ-specific autoimmune diseases which is counteracted by the co-transfer of T_{Reg} cells confirming their suppressive capacities [48]. $RAG^{-/-}$ mice, which are both B and T cell deficient due to non-functional recombination-activated genes, can also be used as recipients for autoimmune-inducing and protection-mediating T cells. Severe combined immunodeficiency (SCID) mice (*scid/scid*) have very few mature B and T cells due to a mutation in a DNA-dependent kinase responsible for the rearrangement of antigen receptors. As recipients these SCID mice develop autoimmune gastritis or a chronic colitis resembling inflammatory bowel disease (IBD) in humans. IBD is not an autoimmune disease per se but is mediated by T cells recognizing antigens derived from the commensal enterobacterial flora. Other models of autoimmune disorders, in which disease development is counterregulated by T_{Reg} cells, should be mentioned. Non-obese diabetic (NOD)

mice possess fewer $CD4^+CD25^+$ T cells in their periphery and develop spontaneous idiopathic autoimmune diabetes, closely resembling type I diabetes in humans. Experimental autoimmune encephalomyelitis (EAE) is a model for human multiple sclerosis which is induced by injection of myelin basic protein and adjuvant [79, 245]. *Scurfy* mice, which are deficient in Foxp3, lack naturally occurring T_{Reg} cells at all and develop a severe autoimmune disorder resembling human IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked; see next chapter).

1.2.2.6.2 Role of T_{Reg} cells in human autoimmunity Despite their critical role in the active suppression of experimental autoimmune disorders, only little is known about T_{Reg} cell involvement in human autoimmunity. In some disorders, a significant decrease in $CD4^+CD25^+$ T_{Reg} cell frequency compared to healthy individuals could be observed, including patients suffering from multiple sclerosis, systemic lupus erythematosus or autoimmune liver disease [37, 38, 80]. Apart from absolute T_{Reg} cell numbers, genetic defects in the $CD4^+CD25^+$ T_{Reg} cell compartment influence pathogenesis of human autoimmune diseases. For example, a mutation in the FOXP3 gene causes the X-linked lymphoproliferative disorder IPEX, which is characterized by autoimmune diseases in multiple endocrine organs. As FOXP3 is generally accepted as a key regulator in $CD4^+CD25^+$ T_{Reg} cells, IPEX is directly linked to a loss of function in this cell type [76]. Furthermore, single nucleotide polymorphisms in various genes such as TNFRSF1B, CTLA4 and CCR5 have been reported to confer high-risk susceptibility to diverse human autoimmune failures like inflammatory bowel disease, type I diabetes and rheumatoid arthritis, respectively [81, 82, 83, 84].

1.2.2.7 T_{Reg} cells in tumor immunity Although $CD4^+CD25^+$ T_{Reg} cells function beneficially *in vivo* to protect the host from the development of autoimmunity, they may also impair anti-tumor immune responses. Although cytotoxic T lymphocytes (CTL) and natural killer (NK) cells that infiltrate and recognize autologous tumors are often identified, effective anti-tumor immunity fails to develop. Since tumor cells largely express self-antigens, T_{Reg} cells may contribute to the immunosuppression of tumor-specific T and NK cell mediated cytotoxicity thus facilitating local tumor growth and metastasis [85]. Increasing evidence supports the existence of elevated numbers of $CD4^+CD25^+$ T_{Reg} cells with potent immunosuppressive activity in peripheral blood (PB) and tumor-infiltrating lymphocytes (TIL) in patients with solid tumors and hematologic malignancies. Beyer and co-workers reported increased frequencies of functional T_{Reg} cells in PB of chronic lymphocytic leukemia patients with decreasing inhibitory function and frequency after fludarabine therapy [41]. Increased percentages of $CD4^+CD25^+$ T_{Reg} cells were also observed in TILs in non-small cell lung cancer (NSCLC), ovarian cancer [86] and in Hodgkin lymphoma [87]. Woo could demonstrate TGF-β secretion, high CTLA4-expression and potent inhibition of T cell proliferation by T_{Reg} cells in NSCLC-TILs thereby providing first evidence for an ac-

tive suppression of anti-tumor immunity by T_{Reg} cells. Furthermore, increased T_{Reg} cell populations were found in PB and TILs in patients with gastric and esophageal [88, 89] as well as in breast cancer [90]. Interestingly, elevated frequencies of $CD4^+CD25^+$ T_{Reg} cells have thereby been correlated with decreased survival rates and poor prognosis [91]. After curative resections, previously increased T_{Reg} cell numbers were significantly reduced while T_{Reg} cell frequency reraised in patients having a relapse [92]. Furthermore, it has been reported that elimination or reduction of $CD4^+CD25^+$ T_{Reg} cells can evoke effective anti-tumor immunity. Targeting T_{Reg} cells by administration of CD25 monoclonal antibody (mAb) abrogated immunologic unresponsiveness to tumors in otherwise non-responding mice by activating tumor-specific cytotoxic T lymphocytes (CTL) and non-specific lymphokine-activated killer (LAK) / natural killer (NK) cells [93, 94]. Combination of T_{Reg} cell depletion and CTLA4 blockade was shown to be synergistic and resulted in maximum tumor rejection [63]. In humans, enhancement of vaccine-mediated anti-tumor immunity in metastatic renal cell carcinoma (RCC) patients was recently demonstrated after depletion of T_{Reg} cells [95]. Thus, removal of T_{Reg} cells might also be useful in vaccination, especially for weakly immunogenic vaccines, resulting in a stronger immune response and a more diverse T cell population.

1.2.2.8 T_{Reg} cells in transplantation tolerance The immune system reacts vigorously to all foreign antigens including allo-antigens presented in transplants. Currently, rejection of MHC-mismatched grafts remains one of the major challenges following transplantation. The predominant approach to ameliorate immunological reactions directed against the allo-organ is the application of immunosuppressive drugs. This therapeutical treatment is complicated by severe side effects, such as increased incidence of serious infections, development of tumors or the inhibition of tolerance in general [76]. As part of their immunosuppressive activity, T_{Reg} cells are able to induce transplantation tolerance thereby contributing to graft survival [42, 44]. Thus, removal of $CD4^+CD25^+$ T_{Reg} cells from wildtype mice was shown to result in enhanced graft rejection, whereas an increase in T_{Reg} cell frequency led to a significantly prolonged graft survival [46]. In order to investigate involvement of T_{Reg} cells in transplantation tolerance in more detail, adoptive transfer studies were performed by several research groups. It was demonstrated that $CD4^+CD25^+$ T_{Reg} cells isolated from animals with long-term surviving allografts can regulate rejection of skin allografts mediated by $CD4^+CD45^{high}$ effector cells from naive mice [97]. These studies indicate that maintenance of allografts is associated with T_{Reg} cell function and can be adoptively transferred to secondary syngeneic hosts. Although the function of human T_{Reg} cells in clinical transplantation has not been clarified, it was shown that human T_{Reg} cells do not only regulate acceptance of solid organ transplants, but can also ameliorate or even prevent graft-*versus*-host disease (GvHD) [96]. Besides infections, severe GvHD is the most relevant complication after allogeneic stem cell transplantation. Immunologically, GvHD is characterized by an expansion of donor lymphocytes with cytotoxic reactivity

against recipient histocompatibility antigens. The resulting clinical picture includes life threatening destruction of skin, gut and liver tissue as the GvHD target organs. In GvHD the transferred immune system lacks the capability to gain control over alloreactive T cell clones. In different murine models, T_{Reg} cells were demonstrated to delay or even prevent GvH-reactivity. Consistent to this, selective depletion of T_{Reg} cells led to an increased severity of acute GvHD *in vivo* [98]. In contrast to that, available data for humans are ambiguous: On the one hand, elevated frequencies of $CD4^+CD25^{high}$ T_{Reg} cells in the peripheral blood of chronic GvHD patients were reported [101], while on the other hand significantly decreased FOXP3 mRNA expression levels were observed in patients suffering from allogeneic or autologous GvHD [102]. A very recent study, analysing the number of infiltrating $CD4^+CD25^+$ $FOXP3^+$ T_{Reg} cells in the intestinal mucosa, found an insufficient up-regulation of T_{Reg} cells in intestinal GvHD lesions [98]. Although the precise mechanisms in the establishment and maintenance of peripheral allograft tolerance remains poorly understood, T_{Reg} cells seem to play a crucial role in this process and might be a powerful tool for clinical application in the prophylaxis of GvHD.

1.2.2.9 T_{Reg} cells in allergy Allergic diseases are chronic inflammatory disorders caused by aberrant immune responses of T_H2 cells against common "innocuous" environmental antigens (allergens) in susceptible individuals. As T_{Reg} cells are able to regulate T_H2 cell responsiveness it has been suggested that genetic and/or environmental factors impairing T_{Reg} cell development and/or suppressor function may contribute to the pathogenesis of allergic diseases. Recent studies have demonstrated that naturally occurring and antigen-induced IL10 producing T_{Reg} cells have a physiological role in protection against human allergies. Individuals with IPEX syndrome, caused by a mutation in the FOXP3 gene, suffer not only from diverse autoimmune but also allergic pathologies including severe eczema, increased serum IgE levels, eosinophilia, and food allergies. Furthermore, PBMCs from allergic patients were shown to generally proliferate more extensively and to produce more T_H2 cytokines in response to allergen stimulation than PBMCs from healthy individuals. Reversly, depletion of $CD25^+$ T cells from control PBMC cultures significantly enhanced immune responses, suggesting active T_{Reg} cell mediated suppression of allergen-specific responses in healthy individuals. Additional studies propose a central role of IL10 and thereby IL10 producing T_R1 cells in the control of allergy, as this cytokine inhibits activation of many cell types and effector functions associated with allergic disorders. The frequency of allergen-specific, IL10 secreting T cells was significantly increased in healthy controls compared with allergic patients, while the reverse was true for T cells producing the T_H2 cytokine IL4. Similarly, it has been demonstrated that IL10 levels are inversely correlated to the severity of human allergic disease. Finally, therapies known to be beneficial for the treatment of allergy and asthma, such as immuno- or glucocorticoid therapy, have been shown to increase IL10 synthesis by T cells. It was additionally

demonstrated that allergen-specific T_H2 cells from allergic patients are less sensitive to inhibition by $CD4^+CD25^+$ T_{Reg} cells suggesting a loss of inhibitory T_{Reg} cell function at the disease site. IL6, produced by activated DCs, was proposed to contribute to this impaired regulatory activity and elevated IL6 levels were indeed reported in asthmatic patients. Blockade of IL6 was demonstrated to evoke local expansion of $CD4^+CD25^+$ $FOXP3^+$ T_{Reg} cells in the mouse lung showing an increased suppressive capacity. Thus, IL10 and IL6 emerge as two cytokines contributing to $CD4^+CD25^+$ T_{Reg} cell activity in allergic disease [255].

1.2.2.10 T_{Reg} cells in clinical use T_{Reg} cells have been demonstrated to play an active role in the prevention of autoimmune disorders as their removal or absence may lead to disease development. But furthermore, T_{Reg} cells are known as important mediators tuning the strength of immune responses. These features render T_{Reg} cells a potential therapeutic intervention tool not only restricted to prevention and/or treatment of autoimmunity but in a large number of disease scenarios involving inappropriate immune responses, such as allergy, allotransplantation, cancer and vaccination [79]. Autoimmune diseases, allergy, allograft rejection and graft-*versus*-host disease (GvHD) in connection with transplantation are conditions reflecting an exaggerated immune response. In these cases, increasing the numbers of T_{Reg} cells could be beneficial which could be reached by two imaginable strategies: The first is to raise T_{Reg} cell frequency *in vivo* by administration of drugs, cytokines or co-stimulatory molecules. The second possibility is to expand T_{Reg} cells *in vitro* by polyclonal activation and IL2 addition followed by their transfer back to the patient. If the antigen responsible for the disease is known, antigen-specific T_{Reg} cell activation could be carried out. Alternatively, T_{Reg} cells can be induced from naive $CD4^+$ T cells *in vitro* by the forced expression of FOXP3 [79]. As in cancer the immune response towards the tumor appears too weak, tumor immunity might be enhanced by T_{Reg} cell depletion *in vivo*. In two murine models, this strategy was successfully applied using anti-CD25 mAb [93, 94]. In a rat colon cancer model, administration of cyclophoshamide destroyed T_{Reg} cells and delayed the outgrowth of tumor. Combining this agent and immunotherapy even cured the rats [246]. Cyclophoshamide not only reduced numbers of T_{Reg} cells but also led to diminished function, enhanced apoptosis and decreased homeostatic proliferation. As recently shown by Dudley *et al.*, cyclophosphamide might be successfully integrated into chemoimmunotherapy. The combination of adoptive transfer of *ex vivo* activated tumor-specific T cells to patients with lymphopenic melanoma after chemotherapy with cyclophosphamide and fludarabine induced tumor-regression in up to 50% of treated patients [247]. In RCC patients, removal of T_{Reg} cells by a recombinant IL2 diphteria toxin conjugate resulted in enhanced vaccine-mediated anti-tumor immunity [95]. Thus, removal of T_{Reg} cells might also be useful in vaccination, especially for weakly immunogenic vaccines, resulting in a stronger immune response and a more diverse T cell population. Approaches combining T_{Reg} cell depletion with other immunologic

interventions, such as transfer of activated T cells or DC-based vaccinations, might be a more beneficial strategy in cancer therapy [63].

Given the central role T_{Reg} cells seem to possess in balancing immune responses, their manipulation could cause severe complications. For example, T_{Reg} cell depletion resulting in tumor immunity might lead to autoimmune diseases, while enhanced T_{Reg} cell frequency and/or activity in order to control autoimmunity might at the same time impair generation of a proper immune response to foreign pathogens. The fine balance between benefit and harm of manipulating T_{Reg} cells was elegantly demonstrated by Antony and co-workers. Transfer of a mixture of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells prevented effective adoptive immunotherapy of established melanoma. In contrast, adoptive transfer of $CD4^+CD25^-$ T cells together with tumor- as well as self-reactive $CD8^+$ T cells into $CD4^+$ T cell deficient host followed by vaccination induced both regression of established melanoma but also severe and undesired autoimmunity [248]. Similarly, depletion of T_{Reg} cells with CD25 mAb in a mammary gland tumor model resulted in tumor regression but significantly increased susceptibility to autoimmune thyroiditis [249]. Another hurdle for T_{Reg} cell application in cancer therapy is the finding that T_{Reg} cell depletion is only transiently maintained, with most cells returning after two months [95]. Caution should therefore be exercised for potential future use of T_{Reg} cells in patients, but nevertheless, manipulation of T_{Reg} cells in terms of their frequency and functional activity appears to offer an attractive and challenging approach in human medicine [79].

1.3 Diseases

1.3.1 Renal cell carcinoma

Renal cell carcinoma (RCC) is an aggressive urologic tumor with a high propensity for metastasis and accounts for approximately 3% of all human malignancies. Its incidence has increased steadily in recent decades. In 2000, 30,000 new cases were diagnosed in the USA and more than 20,000 in the European Union. Annual mortality-to-incidence ratio with RCC is significantly higher compared to other urological malignancies. It is estimated that 25-30% of all patients have metastases at first presentation - due to a lack of symptoms at the onset of disease. Even following complete resection of the primary tumor by radical nephrectomy, the mainstay of therapy for patients with localized disease, relapse occurs in 20-30% of patients. Those who present with metastases have a 5-year survival of less than 10%, whereas the overall 5-year survival rate is 60%.

Metastatic RCC remains one of the most treatment-resistant human malignancies being insensitive to traditional cytotoxic agents as well as radiotherapy. Until today, the most effective agents used are recombinant cytokines, with single-agent interferon (IFN) or interleukin-2 (IL2) showing objective response rates in the 10-20% range. Combination therapies of IFN-α and IL2 with or without additional chemotherapy increase reponse rates to 20-35%, and most responses occur in patients with pulmonary or soft tissue metastases. However, responses are predominantly partial remissions of short duration. Since very recently, standard treatment for RCC patients was extended to the administration of receptor tyrosin kinase inhibitors (e. g. Sorafenib and Sunitinib). Both agents act by blocking signal transduction via the VEGF receptor, while Sorafenib additionally targets the RAF/RAS/MEK/ERK pathway. Until today, there is no standard treatment for patients who fail both therapies resulting in a multitude of experimental second- and third-line therapeutic regimens published during the last decade. Among them, especially the application of monoclonal antibodies also targeting VEGF signaling as well as additional receptor kinase inhibitors have raised well-funded hope as novel approaches in second-line treatment of patients with metastatic RCC [103].

RCC is one of the tumors responding well to immunotherapy. Spontaneous remissions occurring in 0.1 - 1% of RCC patients presenting with lung metastases characterize RCC as an immunogenic tumor. High-dose IL2 therapy has been approved for the treatment of metastatic RCC since 1992. While the overall response rates are relatively low, complete clinical responses appear to be durable in the majority of patients. The mechanism of therapeutic IL2 activity *in vivo* is still not well understood. This is particularly perplexing given recent findings suggesting that IL2 regulates the homeostasis and activation of T_{Reg} cell suppressor function. T_{Reg} cells express all three subunits of the IL2 receptor (IL2R) consisting of one α-chain (CD25), a β-chain (CD122) and a common cytokine receptor γ-chain (CD132). T_{Reg} cells do not secrete IL2 and there-

fore, depend on exogeneous IL2 for survival and function. IL2 knockout mice are characterized by significant impairment in T_{Reg} cells resulting in the development of severe autoimmunity that can be reversed by reconstitution of T_{Reg} cells from normal mice. Increased numbers of T_{Reg} cells were implicated in a variety of human cancers to prevent induction of effective anti-tumor immunity and were additionally correlated to poor prognosis [198]. T_{Reg} cells from cancer patients were demonstrated to possess potent immunosuppressive capacity comparable or even higher than those observed in healthy donors. They suppress proliferation of $CD4^+CD25^-$ T cells and effectively inhibit NK-cell mediated cytotoxicity [85]. As increasing evidence proposes T_{Reg} cells to contribute to tumor immune evasion and given the fact that selected RCC patients benefit from IL2 therapy affecting these cells led us to have a closer look on T_{Reg} cells in RCC patients.

1.3.2 Inflammatory bowel disease

The intestinal immune system is a very large and complex part of the immune system, which interfaces with a variety of endogenous and exogenous stimuli. As the gut mucosa encounters more antigens than any other part of the body, clear discrimination between invasive pathogens and harmless antigens, such as those derived from food and commensal bacteria, is indispensably required. A disturbed balance between tolerance and active immunity may consequently lead to severe inflammatory disorders, among them inflammatory bowel disease (IBD). Apart from T cell anergy and clonal deletion by apoptosis, active suppression by T_{Reg} cells contributes to the maintenance of immunological tolerance towards self- and entero-antigens [193]. Transfer of T_{Reg} cells cannot only prevent the development of colitis in animal models but also cure established disease, acting both systemically and at the site of inflammation. Importance of T_{Reg} cells in the maintenance of intestinal homeostasis is further emphasized in IPEX patients, which suffer from a deficiency in T_{Reg} cells due to impaired FOXP3 function resulting in multiorgan immune disorders, including severe intestinal lesions [74].

Two main forms of IBD exist: Crohn's disease (CD) and ulcerative colitis (UC) are both chronic inflammatory disorders afflicting 0.3% of the Western population. CD can affect any part of the gastrointestinal tract, from the oral cavity to the anus, whereas UC is restricted to colon and rectum. A transmural, granulomatous inflammatory process associated with T_H1-type responses is characteristic in CD, whereas inflammation in UC tends to be limited to the mucosa and contains large numbers of immunoglobulin-secreting plasma cells that appear to be associated with T_H2 responses [239]. Etiology of CD and UC remains poorly understood, but probably involves a complex interplay of environmental factors, genetic susceptibility and abnormalities in immune regulation. IBD is characterized by an exaggerated mucosal immune response thought to be triggered by bacterial antigens, underlined by a heavy influx of T cells, B cells, monocytes and neutrophiles into the intestinal mucosa [9, 105, 193]. The main problem in IBD is a loss of tolerance towards the autologous bacterial flora reflected in a disturbed

interaction between commensal bacteria and the intestinal epithelium. For example, lack of inhibition of TLR signaling can lead to a breakdown of tolerance by induction of costimulatory molecules on DCs and IL6 production resulting in blocked suppressor activity of T_{Reg} cells [192]. On the simplest level, an imbalance between pro- and anti-inflammatory mediators leads to chronic inflammation in the gastrointestinal tract of IBD patients. Specific cytokines important for the induction of mucosal immunity and regulation of the mucosal immune responses include IL1, IL6, IL12 and TNF-α, which are all pro-inflammatory mediators produced by monocytes and macrophages. Additionally, $CD4^+$ T cells infiltrating the lamina propria (LP) of IBD patients display an altered cytokine profile compared to healthy individuals. LP-derived $CD4^+$ T cells from CD patients produce elevated levels of IFN-γ and IL2, whereas $CD4^+$ T cells from the LP of UC patients secrete increased amounts of IL4 and IL5. These observations suggest that the immune responses are T_H1 and T_H2 skewed in CD and UC patients, respectively [193]. This cytokine secretion attracts inflammatory cells like neutrophils and macrophages, which in turn produce a number of mediators, such as leukotrienes, platelet-activating factor and reactive oxygen intermediate which finally lead to the destruction of gut epithelial cells. Defects in apoptosis of activated immune cells may result in the chronification of the inflammatory reaction and may launch a vicious cycle [192].

Fortunately, a number of different medications are available that help to treat IBD symptoms and to keep the disease in remission. The most common therapeutical regimen involves 5-aminosalicylic acid (5-ASA), that reduces inflammation of the intestinal wall. Glucocorticosteroids are applied in a second-line strategy to control the inflammation, when 5-ASA failed. Furthermore, different immunosuppressive drugs may be considered to dampen the inflammatory response. Among them, Infliximab, a monoclonal antibody against TNF has proven to be most effective. Alternatively, microbiological treatment options such as application of "probiotics" (e.g. *Escherichia coli* NISSLE or lactobacilli) may have a beneficial effect [192].

1.4 Aim of this project

T_{Reg} cells are involved in the control of autoimmune diseases as well as anti-tumor immunity and influence the outcome of infections. Despite recent advances in T_{Reg} cell research, requirements for their development, maintenance, and mode of action especially in humans remain poorly understood. Microarrays have illustrated their potential to unravel gene expression in various subsets of leukocytes. Furthermore, we and others have successfully applied this technology to create signatures of murine regulatory T cells in different mouse models, contributing to a better understanding of the mechanisms underlying T_{Reg} cell mediated tolerance and autoimmunity [15, 256, 257]. Thus far, these genomic studies on T_{Reg} cells have been restricted to murine systems. However, differences between humans and mice are highly suggestive and may present obstacles in the transfer from mouse models to actual human disease [258]. These facts led us to direct the aim of this project towards an improved molecular characterization of human T_{Reg} cells in health and disease by the development and application of a non-redundant customized microarray. Therefore, genes should be carefully selected from whole genome expression data previously obtained from regulatory and naive T cells of mouse and human origin, FOXP3 transduced $CD4^+$ T cells and extensive literature search. To gain reliable transcriptional profiles, it is crucial to precisely evaluate the performance of the constructed microarray. T_{Reg} and naive T cells from healthy donors, RCC and IBD patients suffering from distinct stages of disease should be isolated by MACS in adequate purity and appropriately further processed to enable hybridization of individual samples to the microarray. Suitable statistical tools and normalization strategies should be tested to gain the optimal gene expression patterns distinguishing T_{Reg} from naive T cells and to allow comparison of transcriptional patterns between health and disease as well as between different stages of disease. To broaden the knowledge about human T_{Reg} cells, signaling modules underlying T_{Reg} cell function should be extracted by application of pathway analysis and compared between healthy donors, RCC and IBD patients. Furthermore, microarray results of interesting target genes should be verified by quantitative real-time RT-PCR and correlation of mRNA and protein expression should be examined by FACS analyses. As the restricted availability of specific marker molecules is still limiting isolation of human T_{Reg} cells, putative candidates emerging from the obtained expression patterns should be investigated regarding their qualification as new T_{Reg} cell markers. Apart from functional deficits, T_{Reg} cells in disease have additionally been shown to display numerical aberrations, thus also T_{Reg} cell frequencies should be assessed and compared in health and disease.

2 Materials and methods

2.1 Materials

2.1.1 Blood samples and donors' characteristics

Blood samples were collected from 17 healthy donors, 13 RCC and 9 IBD patients after informed consent according to institutional guidelines. The Ethics Committee of Hanover Medical School approved the study protocol. All donors did not suffer from allergies or (additional) autoimmune diseases and were free of lateral or acute infections. Basic characteristics of all volunteers are summarized in Table 1. Mean age was 37±13, 64±10 and 35±11 years for the healthy control group, the RCC and IBD patients, respectively. Altogether, 14 female and 25 male volunteers were analysed.

2.1.2 Primers

Primers used for (real-time) RT-PCR are summarized in Table 2.

2.2 Cell culture

2.2.1 Cell culture conditions

MACS separated human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells were cultured in IMDM + Glutamax I (Gibco, Invitrogen) medium supplemented with 2% of human serum and a final concentration of 50 μg/ml Gentamicin (PAA Laboratories). Cells were incubated at 37°C and 5% CO_2 in the atmosphere.

2.2.2 Freezing and thawing of cells

For later FACS analysis, PBMCs of some donors were sedimented and cell pellets were resuspended in serum-free cell freezing medium (Sigma Aldrich). Afterwards, cells were slowly cooled down to -70°C in special cryo vials using isopropanol. For thawing, cells were shortly incubated in a 37°C water bath and rapidly transferred into appropriate culture medium.

2.2.3 Counting cells

During MACS separation of human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells, cells had to be counted several times in order to calculate optimal antibody and bead concentrations. Cell numbers were determined in a Neubauer counting chamber. Therefore, an aliquot of a diluted cell suspension was mixed with trypanblue, a dye selectively intruding dead cells, while the cytoplasmic membrane of living cells is impermeable for the dye. The number of living cells in 1 ml can be calculated by multiplication of the counted living cells per big square with the dilution factor and 10^4.

- **trypanblue solution:** 0.5 g trypanblue, 0.9 g NaCl, add to 1000 ml water, sterile filtered

donor	age	sex	clinical diagnosis	Array	% purity
healthy donors					
A	58	male	healthy controls	yes	93.9 / 89.2
B	57	female	healthy controls	yes	92.5 / 85.5
C	27	female	healthy controls	yes	83.0 / 69.6
D	27	female	healthy controls	yes	95.5 / 84.8
E	36	male	healthy controls	yes	91.9 / NA
F	39	male	healthy controls	yes	81.9 / 91.1
G	39	male	healthy controls	yes	93.7 / 96.9
H	26	female	healthy controls	yes	97.6 / NA
I	62	female	healthy controls	yes	92.7 / 76.5
J	54	female	healthy controls	yes	98.2 / 91.6
K	26	male	healthy controls	yes	90.3 / 94.3
L	29	male	healthy controls	no	NA / NA
M	30	female	healthy controls	no	NA / NA
N	27	male	healthy controls	no	NA / NA
O	37	male	healthy controls	no	NA / NA
P	31	female	healthy controls	no	NA / NA
Q	23	male	healthy controls	no	NA / NA
RCC patients					
1	71	male	clear cell RCC, localized tumor growth	yes	98.7 / 79.5
2	75	male	clear cell RCC, metastatic tumor growth	yes	93.8 / 86.8
3	63	female	clear cell RCC, localized tumor growth	yes	98.3 / 95.7
4	62	male	papillary RCC, localized tumor growth	yes	97.5 / 85.5
5	61	male	localized tumor growth	yes	99.2 / 83.6
6	65	male	clear cell RCC, localized tumor growth	yes	93.6 / 81.4
7	51	male	clear cell RCC, metastatic tumor growth	yes	93.4 / 91.2
8	50	female	metastatic tumor growth	no	94.2 / NA
9	80	male	metastatic tumor growth	yes	91.7 / 75.2
10	72	male	localized tumor growth	yes	94.7 / 75.1
11	48	male	localized tumor growth, VHL syndrome	yes	89.3 / 86.4
12	68	male	metastatic tumor growth	yes	97.0 / 84.3
13	68	female	metastatic tumor growth	yes	97.9 / 81.9
IBD					
i	29	male	inactive UC	yes	94.5 / 66.7
ii	31	male	inactive UC	yes	95.8 / 63.3
iii	27	male	inactive UC	yes	97.1 / 70.6
iv	44	male	active MC	yes	95.8 / 66.7
v	25	male	active MC	yes	NA / NA
vi	29	female	active MC	yes	95.8 / 85.0
vii	60	female	inactive MC	yes	95.2 / 72.2
viii	40	female	inactive MC	yes	97.9 / 58.4
ix	29	male	inactive MC	yes	96.5 / 71.3

Table 1: Donors' characteristics.

Column %purity summarizes achieved purities of $CD4^+CD25^+$ regulatory/$CD4^+CD25^-$ naive T cells after MACS separation from PBMCs as assessed by FACS analysis.
NA = not assessed

Genes	Primer sequences	product length	5':3' primer [nM]	Primer-Annealing
FOXP3	5'-GAA CGC CAT CCG CCA CAA CCT GA-3' 5'-CCC TGC CCC CAC CAC CTC TGC-3'	224 bp	900 / 900	58°C
CTLA4	5'-TGC AGC AGT TAG TTC GGG GTT GTT-3' 5'-CTG GCT CTG TTG GGG GCA TTT TC-3'	128 bp	50 / 900	58°C
CCR5	5'-CCC GTA AAT AAA CCT TCA GAC CAG-3' 5'-AGG CGA AAA GAA TCA GAG AAC AGT-3'	92 bp	900 / 300	54°C
CCR7	5'-TGG CCT GCA GGA AAC ACC-3' 5'-GGG AGA CTT CTT GGC TTG GTG AG-3'	105 bp	300 / 300	54°C
CCR10	5'-CAC TCC CGG CCG CGC ACA CTT G-3' 5'-GCG CTC GCC CCC TTC ACC GTC GT-3'	165 bp	900 / 300	64°C
TNFRSF1B	5'-GTA GCC TTG CCC GGA TTC TGG-3' 5'-ACC CTG CCC CTG CTC TGC TA-3'	118 bp	300 / 300	58°C
TRAF1	5'-GGG GCA TAA ACT TTC CTC TCC C-3' 5'-TTT GGG GTT ATA CAT TGC TCA GTG-3'	144 bp	300 / 50	58°C
LGALS3	5'-CCT TTG CCT GGG GGA GTG GTG-3' 5'-TGA AGC GTG GGT TAA AGT GGA AGG-3'	130 bp	300 / 300	58°C
RPS9	5'-CGC AGG CGC AGA CGG TGG AAG C-3' 5'-CGA AGG GTC TCC GCG GGG TCA CAT-3'	92 bp	50 / 50	54-64°C

Table 2: Primer characteristics.

2.2.4 *In vitro* activation

For the *in vitro* activation assay, 24-well plates were coated with anti-CD3 (TR66) in a final concentration of 1 μg/ml in PBS containing 50 μg/ml Gentamicin (PAA Laboratories) over night at 4°C. Afterwards this solution was removed and plates were once washed with culture medium. $6.5{\cdot}10^5$ MACS separated T_{Reg} and 10^6 naive T cells each were incubated in appropriate culture medium supplemented with or without 100 U recombinant human IL2 (Proleukin; provided by P Wagner, Ciron Corporation, CA, USA) at 37°C and 5% CO_2. After three and five days, activation status of the cultured cells was confirmed and cells were harvested for real-time RT-PCR or FACS analyses.

2.2.5 Proliferation and suppression assay

T cell proliferation was assessed by stimulating $3{\cdot}10^4$ T cells in triplicate with irradiated allogeneic LG2-EBV B cells or 1μg/ml plate-bound anti-CD3 (TR66) with or without 100 U recombinant human IL2 (Proleukin; provided by P Wagner, Ciron Corporation, CA, USA) in 96-well flat bottom microtiter plates (Nunc). To determine their suppressive activity, $CD4^+CD25^+$ T_{Reg} cells and $CD4^+CD25^-$ naive T cells were cultured under the same conditions but in additional presence of equal numbers of indicated responder T cells. Cells were pulsed with 1μCi/well of ^{3}[H]thymidine after 72 hours for the final 16 hours. Statistical analysis was performed using two-sided Student's *t*-test.

2.3 Methods of molecular biology

2.3.1 Isolation of total-RNA

For cell lysis, human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells were divided into aliquots of maximum $5{\cdot}10^6$ cells, centrifuged for ten minutes at 300 g and 4°C, freed from the supernatant and resuspended in 350 μl RLT-buffer (Qiagen) containing 1% of β-mercaptoethanol (Sigma Aldrich). RNA was extracted from both T cell populations applying the RNeasy kit following the manufacturer's instructions (Qiagen, Germany).

2.3.2 Enzymatic DNA digestion

As DNA contaminating the total-RNA was demonstrated to interfere with subsequent cDNA and cRNA synthesis, a DNA digestion step was added. Therefore, the RNase-free DNase set (Qiagen, Germany) was applied according to the proposed protocol, where the DNA is digested during RNA isolation directly after binding of the sample to the RNeasy column using DNase I.

2.3.3 Photometric determination of nucleic acid concentration and purity

Concentration and purity of the obtained total-RNA, cDNA and cRNA was determined photometrically. Therefore, samples were diluted using TE (10/1) and their optical density (OD) was assessed at a wavelength of 260 nm, which is the absorbance maximum of nucleic acids. An OD_{260nm}= 1 corresponds to approximately 40 μg/ml RNA and 50 μg/ml DNA for a cuvette with 1 cm edge length. Measuring the sample's OD at 280 nm gives information about protein contaminations, as the ratio of OD_{260nm}/OD_{280nm} should lie between 1.8 and 2.0 for protein-free nucleic acid preparations [252].

- **photometer:** BioPhotometer, Eppendorf
- **TE (10/1):** 10mM Tris-HCl (pH 7.4), 1mM EDTA (pH 8.0)

2.3.4 cRNA probe synthesis for microarray analysis

After extraction of total RNA from human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells, its integrity and quality was controlled by running all samples on an Agilent Technologies 2100 Bioanalyzer. Samples were prepared by applying a double-linear amplification method in accordance with the Eberwine protocol [251] and modified by Affymetrix as described briefly in the following.

2.3.4.1 First round of cDNA synthesis Total RNA extracted from $1\text{-}2{\cdot}10^5$ (≈100-200 ng) human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells, respectively,

was transformed into double stranded cDNA in the first step of the amplification protocol [Figure 4]. Therefore, T7-oligo(dT_{23})-Primers hybridize to the poly(A)-tail characterizing eukaryotic mRNAs, thus ensuring that exclusively mRNAs are functioning as template for the SuperScript II Reverse Transcriptase (Qiagen, Germany). Second strand cDNA synthesis was performed including RNase H, that is partly digesting mRNA in the mRNA-cDNA heteroduplexes formed after first strand cDNA synthesis, thus providing primers for the DNA polymerase I (Invitrogen). Addition of T4-DNA polymerase (Invitrogen) to the double-stranded cDNA finally blunt their ends. Afterwards, the synthesized cDNA was purified by phenol-chloroform-isoamylalcohol extraction using PhaseLock Gel Tubes (Eppendorf) to facilitate phase separation. Addition of NaAc and absolute EtOH precipitated the cDNA, that was subsequently washed twice and resuspended in DEPC-water after drying.

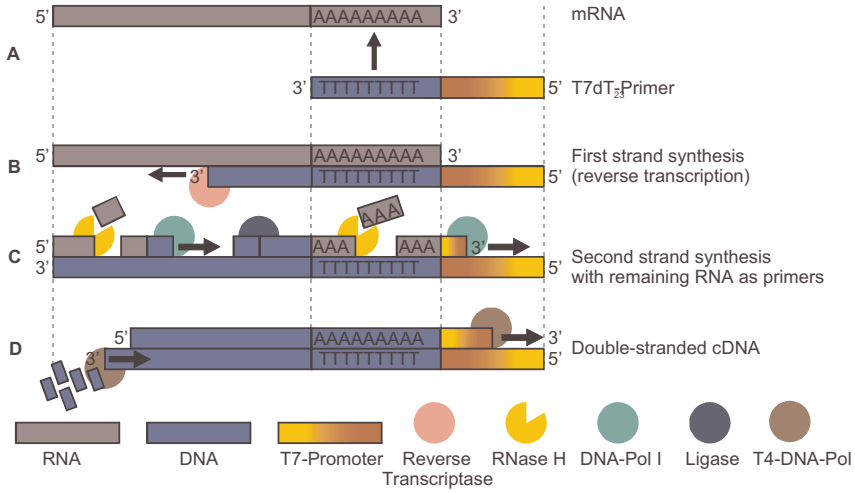

Figure 4: Scheme of the first round of cDNA synthesis.
A. The oligo(dT)-primers selectively anneal to the poly(A)-tail of eukaryotic mRNA. **B.** First strand cDNA synthesis: A RNA-DNA-hybrid is formed by reverse transcription. **C.** Second strand cDNA symnthesis: The RNA is partly digested by RNase H, thus providing the primers for the DNA synthesis by DNA polymerase I. The ligase is linking the synthesized pieces of DNA. **D.** Blunt end generation by T4-DNA polymerase. Adapted from [253].

2.3.4.2 *In vitro* transcription (first round) As all cDNA molecules provide a T7-promotor after cDNA synthesis, they can be transcribed into cRNA by a T7-RNA polymerase [Figure 5]. Therefore, we used the Ribomax^{TM} Large Scale RNA Production System T7 Kit (Promega) enabling the first round of linear RNA amplification without biotinylated nucleotides as each cDNA can repeatedly function as template for the T7-RNA polymerase. The synthesized cRNA molecules are antisense orientated compared to the primary mRNA and lack the T7-promoter, as it is omitted from transcription. The cRNA was extracted from the reaction batch applying the RNeasy kit and its purity and concentration was determined photometrically and by running on the Bioanalyzer.

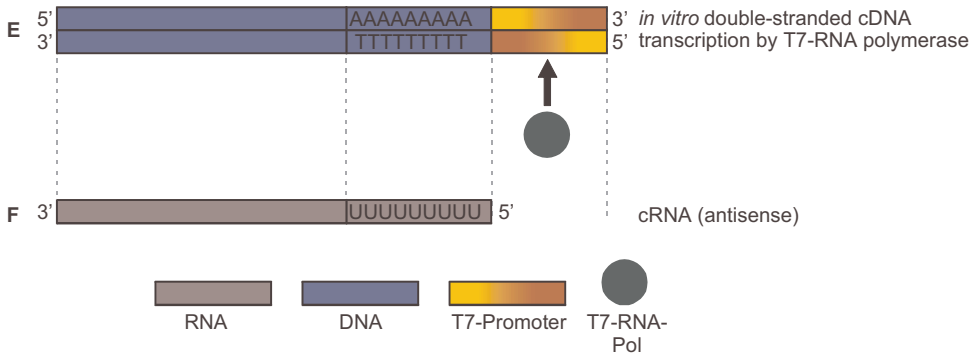

Figure 5: Scheme of the first *in vitro* transcription.
E. The T7-RNA polymerase recognizes the T7-promoter and transcribes the previously synthesized double-stranded cDNA. During this step, the first linear amplification of the starting mRNA takes place. **F.** The resulting cRNA is antisense orientated compared to the primary mRNA. Adapted from [253].

2.3.4.3 Second round of cDNA synthesis For the second linear amplification, the first cRNA was subjected to a second round of cDNA synthesis resembling the first round, but instead of using oligo-dT-Primers, random hexamers were added to the reaction [Figure 6].

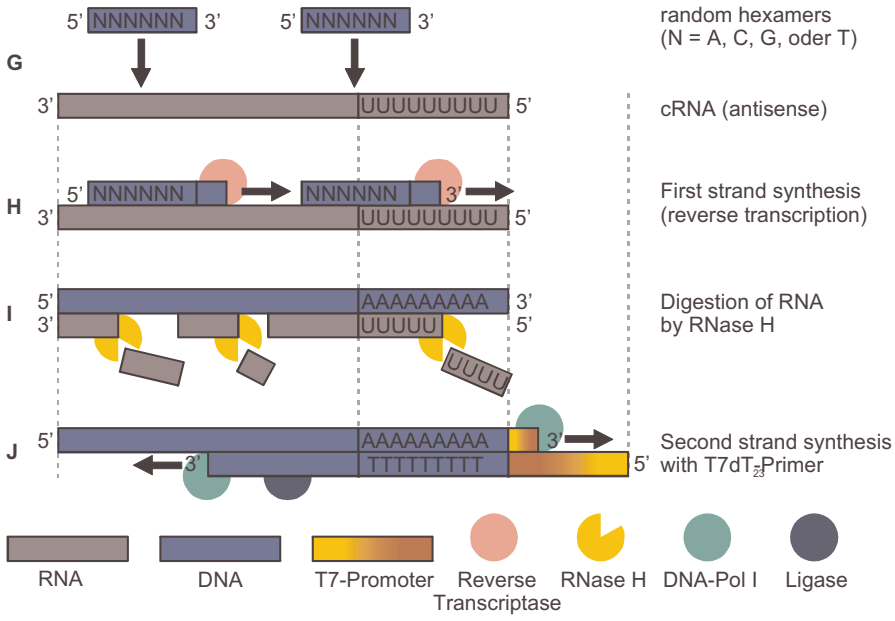

Figure 6: Scheme of the second round of cDNA synthesis.
G and H. Random hexamers anneal to the first cRNA and serve as primers for the Reverse Transcriptase during the first strand cDNA synthesis. **I.** Total digestion of the cRNA strand of the resulting cDNA-cRNA heteroduplex to allow hybridization of the T7dT$_{23}$-Primers. **J.** Second strand cDNA synthesis by DNA polymerase I. [253].

After first strand cDNA synthesis, RNase H functions to digest the whole cRNA strand, enabling annealing of the T7-oligo(dT$_{23}$)-Primer to the poly(A)-tail of the new cDNA strand for second strand cDNA synthesis. Commitment of DNA polymerase I and T4-DNA polymerase resulted in the synthesis of blunt end, double-stranded second cDNA, that was purified and precipitated as described for first round.

2.3.4.4 *In vitro* transcription (second round) including labelling reaction

The second round of linear RNA amplification was performed as an *in vitro* transcription (IVT) assay in the presence of biotinylated UTP using the GeneChip^{R} Expression 3'-Amplification Reagents Kit for IVT Labelling (Affymetrix). Similar to the first amplification round, a T7-RNA polymerase (MEGAscript, Affymetrix) used the T7-promoter provided by the T7-oligo(dT_{23})-Primer for cRNA synthesis, but incorporated *biotinylated* UTP enabling parallel cRNA labelling and later detection [Figure 7]. The synthesized second cRNA was purified and precipitated as described before. The concentration of the biotinylated cRNA was determined by UV absorbance and its quality as means of product length distribution was again checked using the Bioanalyzer.

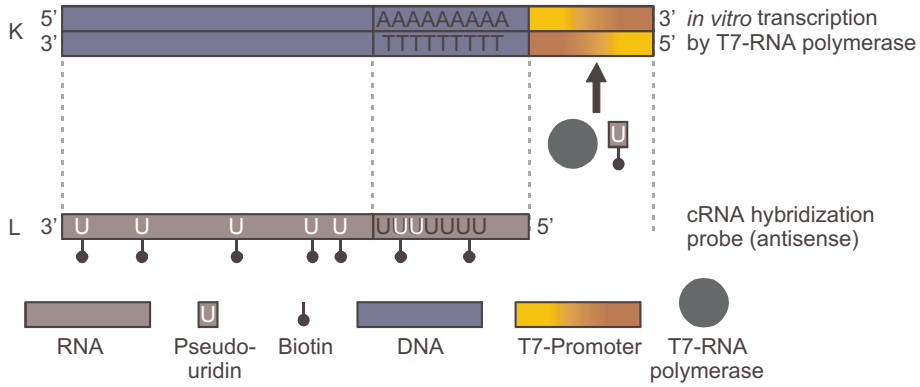

Figure 7: Scheme of the second *in vitro* transcription.
K. T7-RNA polymerase synthesizes antisense cRNA starting from the T7-promoter provided by the cDNA. During this step, the second linear amplification of the starting mRNA take place. **L.** Incooperation of biotinylated pseudo-uridine results in synthesis of biotin labelled cRNA enabling easy detection upon hybridization to the microarray. Adapted from [253].

2.3.4.5 Fragmentation

Each microarray experiment crucially depends on the efficiency, with which the biotin-labelled cRNA sample molecules bind to their immobilized targets. Due to hybridization kinetics, this efficiency improves with decreasing sample length. For similar hybridization efficiencies of all sample molecules, a narrow length distribution needs to be achieved. This was done by fragmentation of the cRNA using a buffer containing high potassium- and magnesium-ion concentrations at high temperatures. Per microarray, 15 μg of biotinylated cRNA were subjected to fragmentation at 94°C for 35 minutes.

- **fragmentation buffer (5x):** 4ml 1M Tris-acetate (pH 8.1), 0.98g K-acetate, 0.64g Mg-acetate, add to 20ml of Milli-Q water, sterile filtered

2.3.5 real-time RT-PCR

To verify accuracy of the obtained microarray expression data, real-time RT-PCR of selected genes was performed. Therefore, $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells were isolated by MACS technology as described below. After cell lysis, RNA was extracted from both cell populations applying the RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized using oligo(dT) primers and random hexamers by SuperScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Quantitative real-time RT-PCR was performed in an ABI PRISM cycler (Applied Biosystems) applying a SYBR green PCR kit from Stratagene and specific primers optimized to amplify 90-230 bp fragments from the different genes analysed. A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach it was calculated for every gene. Relative mRNA levels were determined by using included standard curves for each individual gene and further normalization to RPS9 as a housekeeping gene. Melting curves established the purity of the amplified band. Primer sequences and their characteristics are summarized in Table 2.

2.3.5.1 Primermatrices To prevent amplification of unspecific products and generation of primer dimers, the concentrations of the 5'- and 3'-primers used for the real-time RT-PCR had to be optimized. Therefore, concentrations ranging from 50 nM to 900 nM for each primer were tested and the optimal primer combination resulted from the maximal difference of the C_t-values obtained for the specific amplicon and the negative control [Table 2, column 5':3' primer].

2.4 Immunological methods

2.4.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn blood by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation as described previously [120]. First, the blood samples were diluted 2-4 times with rinsing buffer, carefully applied onto the Ficoll and subsequently centrifuged for 30-40 minutes at room temperature and 400 g without brake. Thereby, mononuclear cells, consisting mainly of lymphocytes and monocytes, are enriched in the interphase. These cells have been sucked off, transferred into a new tube and washed with FACS buffer for ten minutes at 4°C and 300 g. Afterwards, pelleted cells were freed from the supernatant, resuspended in FACS buffer again and centrifuged at 200 g and 4°C for another 10 minutes to remove the thrombocytes. The received cell pellet is mainly composed of PBMCs and was resuspended in 90 μl ice cold FACS buffer per 10^7 cells.

- **rinsing buffer:** PBS, 2mM EDTA
- **FACS buffer:** PBS, 2mM EDTA, 2% FCS
- **Ficoll**: Biocoll Separating Solution, density = 1.077 g/ml, Biochrom AG

2.4.2 Magnetic cell separation (MACS)

For isolation of human $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells, PBMCs were further separated using the Regulatory Human T cell Isolation Kit and an AutoMACS separation unit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. In the first step, non-$CD4^+$ T cells were depleted by indirect magnetic labelling with a biotin-antibody cocktail and anti-biotin MicroBeads [Figure 8A].

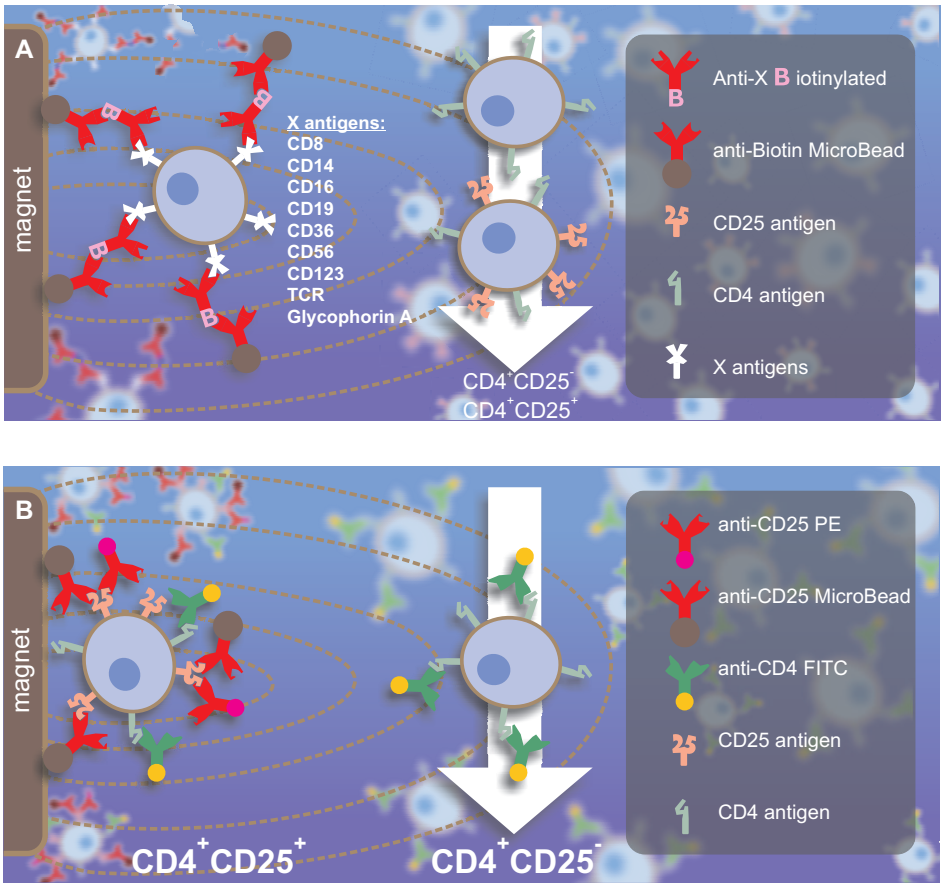

Figure 8: MACS separation strategy [253].
A. Enrichment of $CD4^+$ T cells from PBMCs. Cells expressing one of the "X" surface antigens are specifically labelled by biotinylated antibodies. Binding of anti-Biotin MicroBeads to these antibodies result in retention of labelled cells in a magnetic field provided by the AutoMACS unit, while unlabelled cells - containing the $CD4^+$ T cell fraction - flow through. **B.** Separation of $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells. $CD4^+CD25^+$ T cells are directly labelled with anti-CD25 MicroBeads and are thus retained in the magnetic field, whereas the unlabelled $CD4^+CD25^-$ cells can pass through. After removal of the magnetic field, the $CD4^+CD25^+$T cells can be eluted from the columns. The indicated CD4-FITC and CD25-PE antibodies enable subsequent analyses of the obtained purities of both T cell subpopulations by FACS.

In the second step, $CD4^+CD25^+$ T cells were directly labelled with CD25 MicroBeads and isolated by positive selection from the pre-enriched $CD4^+$ T cells [Figure 8B]. The magnetically labelled $CD4^+CD25^+$ T cells were retained on the column,

whereas the $CD4^+CD25^-$ T cells flowed through. After removal of the magnetic field, $CD4^+CD25^+$ T cells could be eluted after separation over two columns. To increase purity of the $CD4^+CD25^-$ T cell fraction an additional separation step depleting remaining $CD4^+CD25^+$ T cells was added, if necessary. Purity of the enriched cell fractions was >90% as determined by flow cytometry.

2.4.3 Flow cytometric analysis

To confirm purity of the separated cell fractions, to determine T_{Reg} cell frequency and to verify gene expression data on protein level, PBMCs or separated $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells were analysed by multicolour fluorescence-activated cell sorting (FACS) using the antibodies listed below. Flow cytometry was done on a FACS Calibur applying CellQuest software (BD Biosciences). For immunostaining of surface proteins, cells were incubated in FACS buffer containing appropriate concentrations of the particular antibody at 4-8°C. Intracellular FOXP3 staining was performed as recommended by the manufacturer applying the delivered fixation and permeabilization buffers. Intracellular LGALS3 detection was done after fixation of cells in 4% para-formaldehyde and permeabilization in 0.5% saponin, both in PBS/EDTA. Apoptotic cells were stained with Annexin V and PI according to the protocol provided in the Annexin V-FITC apoptosis detection kit.

- anti-CD4-FITC and -APC (M-T466), anti-CD25-PE (4E3): Miltenyi Biotec
- anti-FOXP3-APC (PCH101) staining set: eBioscience
- monoclonal anti-TNFRSF1B-FITC (22235.311): R&D Systems
- monoclonal anti-LGALS3-FITC and -PE (B2C10): BD Pharmingen
- isotype control antibody (MOPC-21): mouse IgG_1, κ, FITC or PE conjugate: BD Pharmingen
- polyclonal antibody to human CCR10 from goat: Alexis Biochemicals
- rabbit anti-goat IgG secondary antibody, Alexa Fluor 647: Molecular Probes
- Annexin V-FITC apoptosis detection kit: BD Pharmingen
- propidium iodide (PI): 1mg/ml PI in PBS, diluted 1:1000 before use

2.5 Methods of microarray technology

2.5.1 Manufacturing the *Human T_{Reg} Chip*

395 different oligonucleotides were deposited onto CodeLink activated slides (Amersham Biosciences) at a concentration of 25 μM in 1.5 x sodium phosphate buffer in a contact-dependent manner using a MicroGrid TAS II spotter (BioRobotics, Freiburg, Germany). All these 50mers were amino-modified at the 5'-end enabling covalent linkage to reactive ester groups provided by the glass surface. Coupling of DNA was ensured by over night incubation in a saturated sodium chloride chamber and blocking of residual reactive groups was done as recommended by the manufacturer [118]. Until used, slides were maintained in a desiccated environment. To ensure complete spotting, SYBR green staining of three randomly selected *Human T_{Reg} Chip*s of each printing batch was performed as previously described [119].

2.5.2 Design of the *Human T_{Reg} Chip*

Each probe of our microarray consists of a single 50mer oligonucleotide, as utility and performance of 50mer oligonucleotide microarrays were proved before [117]. The *Human T_{Reg} Chip* is composed of 350 oligonucleotides probing genes associated with T_{Reg} cells and 31 oligonucleotides representing housekeeping genes consulted for normalization. Furthermore, lots of control oligonucleotides are included: two 5'-3'-controls to ensure RNA integrity, four bacterial hybridization controls to examine a linear hybridization process, five spike-in controls to check sample preparation, one positive control (*Arabidopsis thaliana*) for simpler grid finding and finally 32 negative controls to calculate the background level. Altogether, we immobilized eight replicates per oligonucleotide, splitted in two separated arrays per slide, each containing 1600 spots. Genes probed on the *Human T_{Reg} Chip* were selected by extensive analyses of literature and previously performed Affymetrix microarray experiments [196]. Design and synthesis of the oligonucleotides was performed by MWG using the Affymetrix probe sets as reference. The *Human T_{Reg} Chip* will be freely available to the scientific community.

2.5.3 Hybridization

In all cases, 15 μg of each biotin-labelled cRNA preparation was fragmented and placed in a hybridization cocktail (Amersham Biosciences) containing four biotinylated hybridization controls (BioB, BioC, BioD, Cre). This hybridization solution buffered the pH, decreased surface tension to facilitate moistening of the microarray slide and contained auxiliary components allowing low hybridization temperatures in order to prevent thermic degradation of cRNAs. Samples were hybidized to individual *Human T_{Reg} Chip*s for 16 hours using a Lucidea Slidepro (Amersham Biosciences).

2.5.4 Posthybridization and Cy5-streptavidin staining

Unbound cRNA and the components of the hybridization cocktails have to be stringently washed off after hybridization in order to ensure low background noise. Thus, slides were washed in 0.75 x TNT at 46°C for one hour. Afterwards, they were stained for 30 minutes at room temperature with Cy5-streptavidin working solution. Streptavidin is a 60 kDa extracellular protein of *Streptomyces avidinii* possessing four high affinity biotin binding sites, thus specifically interacting with the biotinylated UTP of the hybridized cRNA. Excessive Cy5-streptavidin was washed off in four steps by incubation in 1 x TNT for five minutes. Afterwards, slides were dipped into 0.05% Tween for 10 secondes and centrifuged to dry.

- **1 x TNT:** 200 ml 1M Tris-HCl (pH 7.6), 60 ml 5M NaCl, 1 ml Tween20, add to 2000 ml Milli-Q water, sterile filtered
- **TNB buffer:** 522 ml Milli-Q water, 60 ml 1M Tris-HCl (pH 7.6), 18 ml 5M NaCl; heat to 60°C, add 3g of NEN Blocking Reagent (Amersham) in 0.5g increments and let dissolve, stir 30 minutes at 60°C, sterile filter, aliquot and freeze at -20°C; thaw over night at 4°C prior use
- **Cy5-streptavidin stock solution:** 1 mg Cy5-streptavidin (Amersham Biosciences), 1 ml DEPC water; slowly vortex three times, keep on ice for five minutes, freeze aliquots at -20°C
- **Cy5-streptavidin working solution:** prepare 3.4 ml per slide, resuspend 6.8 μl of the Cy5-streptavidin stock solution in TNB buffer

2.5.5 Scanning and signal quantification

Using a laser-fluorescence scanner (arrayWorX^e, Applied Precision) and an excitation wavelength of 694 nm the stained microarrays were read at a solution of 10 μm and an exposure time of one second. Obtained fluorescence signals for all 3200 spots were expressed and saved as image data, that were converted into numerical values using ImaGene software (version 5.5, BioDiscovery). Correlation of the particular spots to the associated oligonucleotide was performed with the aid of an allocation file generated by the spotter. The provided grid and the image are overlayed and the software calculates a signal intensity and a local background intensity for each spot. The difference between the first and the latter yields the effective signal strength of the spot. During this calculation, additional quality flags are assigned to the spots: Good or unflagged spots are indicated zero, signal intensities near the background level are flagged as empty or two, and spots of poor quality (due to e.g. strange morphology or dust on the slide) are tagged with three. During calculation of the array raw data, spots flagged with 3 due to poor quality and spots with signal intensities smaller than the mean signal intensity of the negative controls were excluded from further analysis.

2.5.6 Normalization strategy

Normalization adjusts for differences in labelling and detection efficiencies for the fluorescent labels and for differences in the quantity of initial RNA from the samples examined in one assay. Countless normalization strategies exist. To adjust arrays from different experiments, we finally decided to normalize our data based on median signal intensities of the housekeeping genes as previously proposed [164] and described in chapter 3.2.6 using the following formula:

$$SI_{\text{norm}} = \frac{I_n - B_n}{e^{<ln(house)>}}$$

Where SI_{norm} is the normalized signal intensity, I_n is the signal intensity of gene n, B_n is the background intensity of gene n, and <ln house> is the median logarithmic signal intensity of all housekeeping genes.

2.5.7 Significance Analysis of Microarrays and hierarchical clustering

To ascertain gene expression changes between human $CD4^+CD25^+$ T_{Reg} and $CD4^+$ $CD25^-$ naive T cells differences in gene expression were determined statistically by corrected *t*-test analysis using the SAM tool [5]. For two-dimensional hierarchical clustering analysis Genesis software v1.4.0 was applied [254].

2.5.8 Pathway analysis

Genes that were significantly up- or down-regulated in human $CD4^+CD25^+$ T_{Reg} *versus* $CD4^+CD25^-$ naive T cells from healthy donors were imported into PathwayAssist 3.0.8 (Ariadne Genomics, Rockville, MD). The pathway was built using the "find direct interactions" algorithm based on the embedded ResNet 3.0 database containing 500,000 links for approximately 50,000 proteins, extracted from 4.5 million MEDLINE publications (as of February 26, 2005).

2.5.9 Accession numbers

The entire data set for healthy donors was deposited in a MIAME compliant format at Gene Expression Omnibus (GEO). Data derived from the *Human T_{Reg} Chip* are available under the series accession number GSE3882 (platform ID, GPL 3110). Data derived from the Affymetrix GeneChips used as reference and selection data sets were published at GEO under series accession number GSE4527 (FOXP3 and GFP transduced $CD4^+$ T_H cells) and GSE4571 (representing data from $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells isolated by cell sorting from human peripheral blood and from spleen of BALB/C mice).

2.6 Affymetrix GeneChip experiments

Prior to this study, Affymetrix GeneChip experiments were performed by hybridization of cRNA derived from regulatory and naive T cells of mouse or human origin, from FOXP3 and control vector transfected human $CD4^+$ T_H cell lines and a human $CD4^+CD25^+$ derived T_{Reg} cell line (kindly provided by W. Hansen and M. Probst-Kepper, GBF Braunschweig, Germany, [197]). Samples were amplified according to the recommended protocols of the manufacturer and described above. In all cases, 10 μg of each biotinylated cRNA was fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, Cre). Samples were hybridized to an identical lot of Affymetrix GeneChips for 16 hours. After hybridization the GeneChips were washed, stained with SAPE, and read using an Affymetrix GeneChip fluidic station and scanner. Data derived from these Affymetrix GeneChip experiments were used as reference and selection data sets in this project.

2.6.1 Purification of human and murine $CD4^+$ T cells

Human $CD4^+$ T cells were prepared from peripheral blood of healthy donors by centrifugation over Ficoll-Hypaque gradients (Biochrom AG, Berlin, Germany) and MACS isolation using the $CD4^+$ T cell isolation kit and an AutoMACS unit (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, cells were separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells by sorting on a MoFlo (DakoCytomation, CO, USA) to a purity in excess of 98%. Red blood cell depleted splenocytes from BALB/c mice were labelled with anti-CD4 and anti-CD25 antibodies and also separated with a MoFlo in comparable purities.

2.6.2 Retroviral transduction of human effector $CD4^+$ T cells

The cDNA encoding human FOXP3 was amplified from cDNA of T_{Reg} cells using high fidelity PFU polymerase (Promega) and specific primers (FOXP3: 5'-GAC AAG GAC CCG ATG CCC A-3' and 5'-TCA GGG GCC AGG TGT AGG TG-3'). The PCR product was cloned into pCR4.1 TOPO (Invitrogen, CA, USA), sequenced, and inserted into a pMSCV-based retroviral vector encoding an enhanced GFP under the control of an IRES sequence. The amphotropic PT67 package cell line (kindly provided by M. Wirth, GBF Braunschweig, Germany) was used for transfection. Filtrated (0.45 μm) virus-containing supernatant supplemented with 8 mg/ml sequabrene (Sigma Aldrich, Munich, Germany) was applied to T_H cells at day two after allogeneic stimulation by centrifugation at 5000 x g for 60 minutes at room temperature. Cells were expanded thereafter with 50 U/ml IL2 and GFP-expressing cells were sorted 1-2 weeks later using a FACS-Vantage (BD Bioscience).

2.6.3 Criteria for *Human T_{Reg} Chip* gene selection

Differentially expressed genes between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells of either mouse or human origin measured on Affymetrix GeneChips were selected according to predefined categories deduced from three parameters calculated by MAS 5 software: fold change (FC), change p value (pValue), and signal intensity difference (SID). Category A is defined as a FC above 2, pValue < 0.001 (for increased) or pValue > 0.999 (for decreased), and SID above 200. Category B is defined as a FC above 2, pValue < 0.01 (for increased) or pValue > 0.99 (for decreased), and SID above 100. Category C is defined as a FC above 1.5, pValue < 0.001 (for increased) or pValue > 0.999 (for decreased), and SID above 40. The likelihood of a significant regulation decreases from category A to C. Thus, most of the genes selected for the *Human T_{Reg} Chip* were categorized as A. Selection was performed by collecting genes that were significantly regulated in human cells, genes that were similarly regulated between mouse and human, genes that were found to be regulated only in mouse cells and referenced in the literature, and genes that were significantly affected by FOXP3 overexpression in cultured T_H cell lines. Genes known for their impact in mouse and human regulatory T cell development were also considered [196, additional data files 1-4].

3 Results

3.1 Development and design of the *Human T_{Reg} Chip*

To improve molecular characterization of human T_{Reg} cells, a unique microarray has been compiled, the *Human T_{Reg} Chip,* consisting of 350 T_{Reg} cell associated genes. The oligonucleotides probed on the *Human T_{Reg} Chip* were selected on the basis of whole genome transcription data obtained from human and mouse $CD4^+CD25^+$ and $CD4^+$ $CD25^-$ T cells measured on Affymetrix GeneChips [196, additional data file 1]. $CD4^+$ T cell subsets were isolated from either human peripheral blood or murine splenocytes and separated using FACS-based cell sorting at purities constantly greater than 98%. Differential gene expression was determined using statistical parameters, as described in Material and methods (see chapter 2.6.3). This primary data set was extended to genes that were affected by FOXP3 over-expression in cultured human $CD4^+$ T_H cell lines. Therefore, $CD4^+CD25^-$ derived T_H cell lines were infected with retroviruses encoding for FOXP3 and GFP (green fluorescent protein) under the control of an internal ribosomal entry site (IRES) or with an empty control vector containing only GFP (kindly provided by M. Probst-Kepper, [197]). FOXP3 could partially induce a regulatory phenotype in these transfected cells *in vitro* (data not shown). Using Affymetrix GeneChips, these genetically engineered cells were compared to those infected with the GFP control vector only. Furthermore, a human T_{Reg} cell line was analysed that was derived from human $CD4^+CD25^+$ T cells, maintained its regulatory phenotype *in vitro* and compared its gene expression profile to the control $CD4^+$ T_H cell line (kindly provided by M. Probst-Kepper, [197]). For the development of the *Human T_{Reg} Chip* those genes were added to the primary data set that were differentially expressed in both experiments by more than twofold [196, additional data files 2 and 4]. Moreover, T_{Reg} cell associated genes identified by extensive literature search were included [196, additional data file 3]. In summary, this process resulted in the selection of 350 T_{Reg} cell relevant genes that were arranged on an oligonucleotide microarray. Furthermore, 45 control genes were included in the primary microarray design. Each probe of the microarray consists of a single 50mer oligonucleotide, as utility and performance of 50mer oligonucleotide microarrays were proved before [117]. Design and synthesis of the oligonucleotides was performed by MWG using the Affymetrix probe sets as reference. Altogether, eight replicates per oligonucleotide were immobilized, splitted in two separated arrays per slide, each containing 1600 spots [Figure 9B]. Applying the MicroGrid TAS II spotter, 25μM of each oligonucleotide were deposited in a contact-dependent manner onto CodeLink activated slides (Amersham Biosciences) providing reactive ester groups to covalently link the aminomodified oligonucleotides to the glass surface. Quality control of three randomly selected slides of each chip batch was carried out by SYBR green staining, to ensure complete spotting and that signal values near the background intensity are really due to low transcript levels in the sample [Figure 9A].

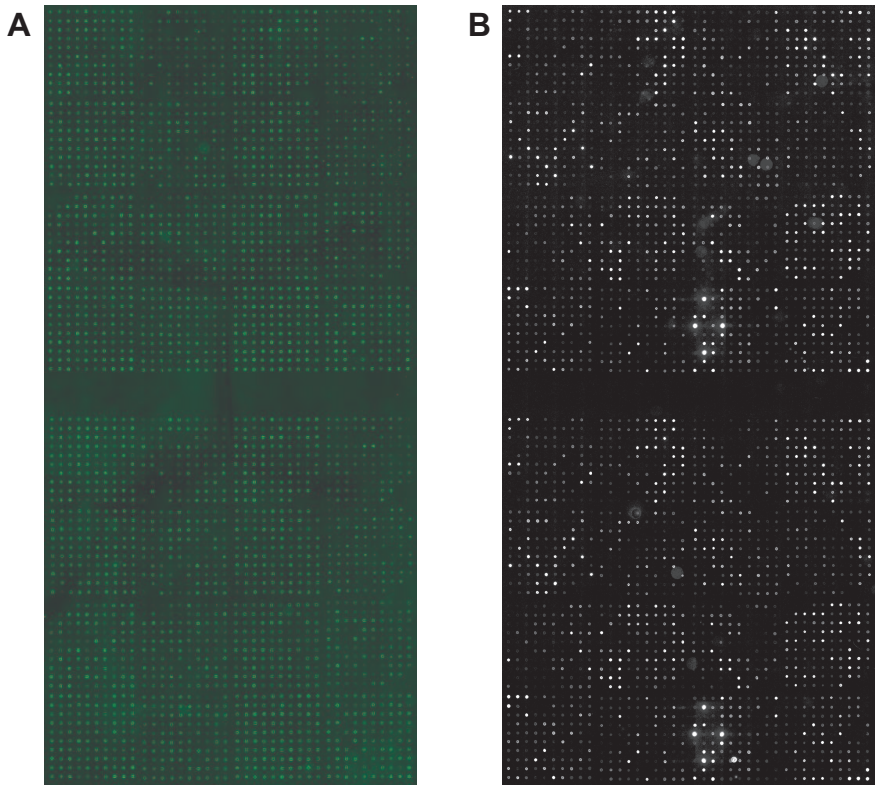

Figure 9: The *Human T_{Reg} Chip*.
A: SYBR green staining as quality control of the spotting process. As SYBR green is interacting unspecifically with the immobilized oligonucleotides, the applied spotting pattern can be demonstrated. A 4 x 4 pin configuration was used, with each pin printing a square of 10 x 10 spots, resulting in 1600 spots per array. Two of these arrays were replicated per slide of the *Human T_{Reg} Chip*. Missing spots in this image are due to negative controls. Furthermore, a homogeneous spot morphology, similar signal intensities (due to the same concentration of all printed oligonucleotides) and low background levels are illustrated. **B:** Representative *Human T_{Reg} Chip* hybridized with cRNA derived from regulatory T cells isolated from a healthy donor. In contrast to the SYBR green staining in A, variation in spot signal intensity can be observed, directly reflecting the different expression levels of the 395 test genes. Additionally, a high intra-array reproducibility is demonstrated, as both replicates show very similar signal intensity patterns.

3.2 Performance and evaluation

Important factors for selecting an appropriate microarray format include cross-platform comparison, sensitivity, specificity and both intra- and inter-assay reproducibility [106]. To ensure measurement of accurate and reliable transcription profiles, all these quality parameters have been validated for the 59 *Human T_{Reg} Chip*s contributing to the expression profile that discriminates $CD4^+CD25^+$ regulatory from $CD4^+CD25^-$ naive T cells isolated from healthy donors.

3.2.1 Comparability to Affymetrix

To evaluate the performance of the *Human T_{Reg} Chip* its comparability to Affymetrix as the market leader of microarray technology was first examined. Therefore, relative expression data gained from experiments investigating FOXP3 affected gene expression on Affymetrix GeneChips were used as reference data in a cross-platform comparison. Identical samples, obtained either from FOXP3 transfected $CD4^+CD25^-$ T cells or GFP transfected controls, were hybridized to Affymetrix HG_U133A GeneChips^R and *Human T_{Reg} Chip*s, respectively. As demonstrated, concordance of significantly regulated genes on both platforms was 81% [29/36; Figure 10]. Opposite regulation was only observed for few marginally regulated genes (7/36).

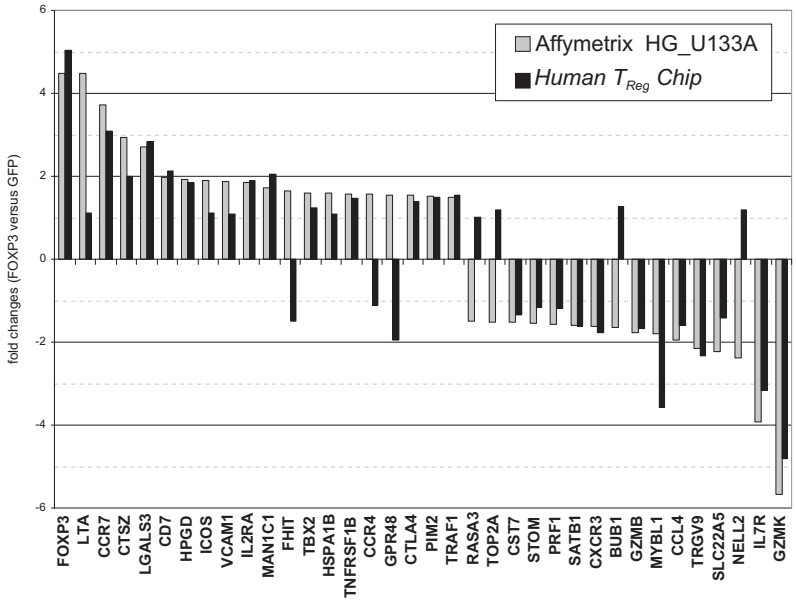

Figure 10: Comparability to Affymetrix.
Splitted samples (FOXP3 or GFP transfected T cells) were hybridized to Affymetrix HG_U133A GeneChips^R and *Human T_{Reg} Chip*s, respectively. Affymetrix genes showing a regulation of at least 1.5fold (excluding signals labelled as absent on both microarrays) were compared to those fold changes arising from the *Human T_{Reg} Chip* platform (including noisy fold ratios generated from low intensity genes). As depicted, 29 out of 36 genes demonstrated similar regulation on the *Human T_{Reg} Chip* compared to Affymetrix, resulting in a correlation of 81%.

3.2.2 Reproducibility of the *Human T_{Reg} Chip*

A basic property of a good microarray platform is high reproducibility in repeated experiments [108]. That is why stability of signal intensities arising from the same sample hybridized to several *Human T_{Reg} Chip*s was assessed. Figure 11 illustrates a scatterplot of normalized signal intensities of two exemplarily selected slides, showing that almost all signals were located along the bisecting line within the two-fold range,

reflecting the relatively low measurement noise in the data. Linear regression for 52 replicated microarray experiments applying the *Human T_{Reg} Chip* yielded a median correlation coefficient of 0.98, demonstrating high reproducibility in measurement, even for low signal intensities.

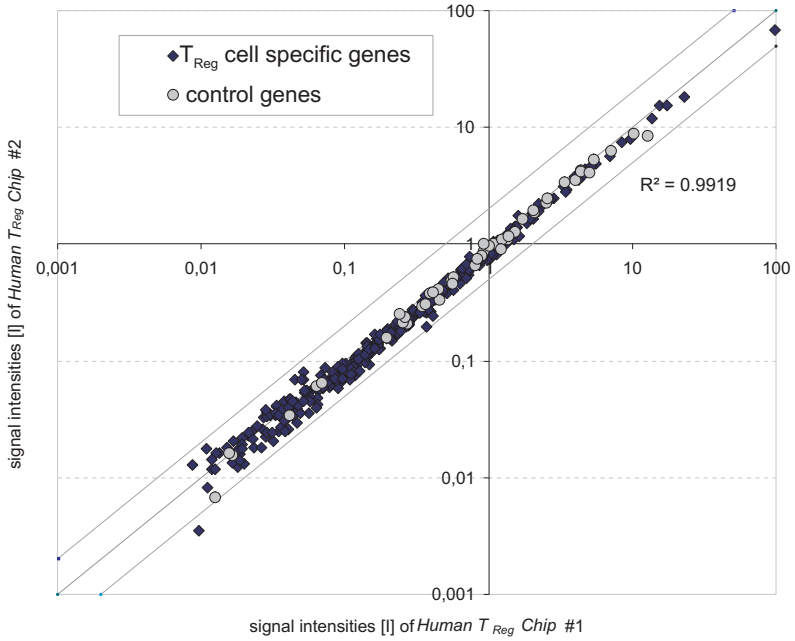

Figure 11: Reproducibility of the *Human T_{Reg} Chip*.
The same sample was hybridized to several *Human T_{Reg} Chip*s. A log-log-plot of normalized signal intensities of two exemplarily selected slides is illustrated, showing that 99.7% of all signals are located within the two-fold range.

3.2.3 Coefficient of variance

As written above, each oligonucleotide is replicated eight times per microarray, enabling the calculation of variance coefficients (CV) for each probed gene as another performance criterion of the *Human T_{Reg} Chip*. The CV is defined as the standard deviation divided by the mean [113]. The pie chart in Figure 12 summarizes mean CV values obtained from the 59 *Human T_{Reg} Chip*s constituting the expression profile of T_{Reg} cells isolated from healthy donors. It is depicted that the vast majority of signal intensities (74%) have a CV < 0.3, meaning that their values vary less than 30% across the entire data set, which is a threshold used for industrially manufactured microarrays.

3.2.4 Quality flags

Other performance parameters consulted for the evaluation of the *Human T_{Reg} Chip* are the so called quality flags. After hybridization the slides are scanned and the resulting images are analysed by ImaGene® (Biodiscovery, El Segundo, CA), a software which

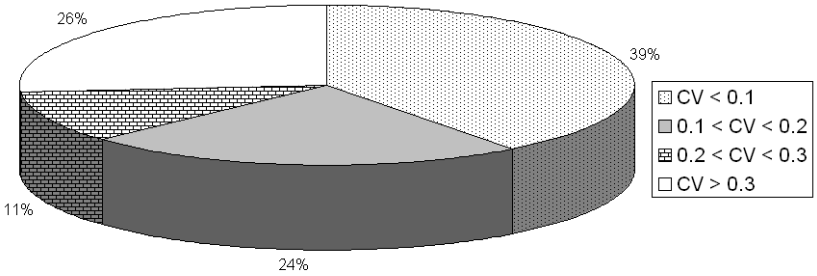

Figure 12: Coefficients of variation (CV).
The ratios of standard deviation and mean were calculated for each gene probed in eight replicates per microarray. CVs of all 59 experiments applying the *Human T_{Reg} Chip* contributing to the expression profile of human T_{Reg} cells from healthy donors are presented as means. As demonstrated, 74% of all signals have a CV < 0.3.

quantifies and qualifies each spot on the array. During this calculation it assigns quality flags to the spots: Good or unflagged spots are indicated zero, signal intensities near the background level are flagged as empty or two, and spots of poor quality (due to e.g. strange morphology or dust on the slide) are tagged with three.

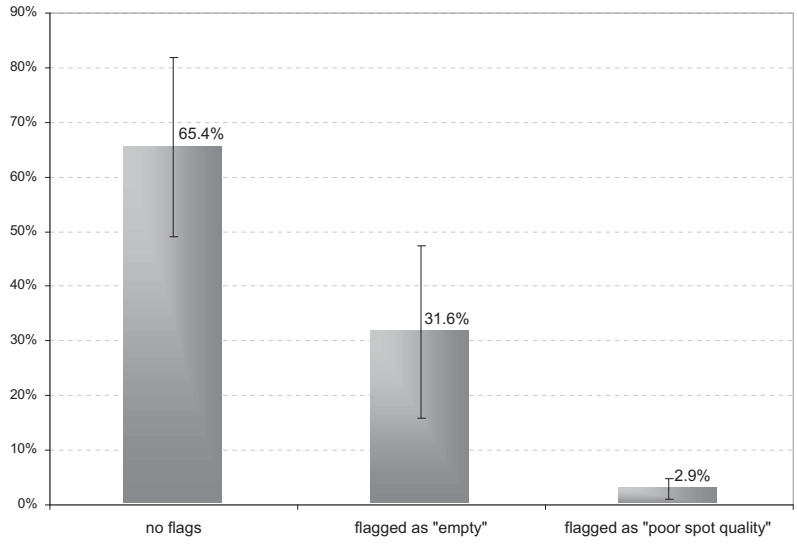

Figure 13: Quality flags.
Quality flags are displayed as assigned by ImaGene^{R} software (v5.5.2). Values represent means of all 59 microarray experiments applying the *Human T_{Reg} Chip* that constitute the expression profile of healthy human T_{Reg} cells. As depicted, almost two third of all spots were left unflagged, 32% have signal intensities near the background level and less than 3% were flagged due to poor spot quality.

In Figure 13, quality flags of the 59 *Human T_{Reg} Chip*s constituting the expression pattern of human T_{Reg} cells isolated from healthy volunteers, are displayed as means calculated across the entire data set. It is demonstrated, that normally 65.4% of all spots are unflagged, 31.6% are flagged as empty (including 2% due to negative controls) and 2.9% are of poor quality.

3.2.5 Linearity of hybridization process

To monitor the hybridization process regarding its linear run and to determine microarray system sensitivity, bacterial hybridization controls were recorded at different concentrations as another performance criterion of the *Human T_{Reg} Chip*. Therefore, normalized signal intensities *versus* concentrations of the used hybridization controls were plotted as means of 59 experiments [Figure 14]. Linear regression yielded a correlation coefficient of > 0.96, demonstrating that a linear hybridization process covering more than three orders of magnitude of concentrations is ensured. Furthermore, this plot reveals a final detectable concentration of 0.3 pM.

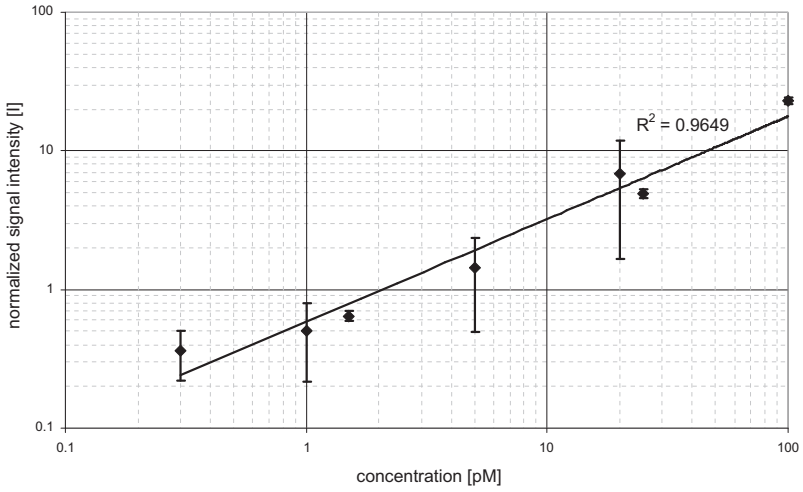

Figure 14: Hybridization controls.
Normalized signal intensities *versus* concentration of used hybridization controls are plotted as means of 10 (1.5 pM, 25 pM, 100 pM) and 59 experiments applying the *Human T_{Reg} Chip*. Standard deviations are visualized by error bars. Linear regression yielded a correlation coefficient of > 0.96, demonstrating a linear hybridization process covering more than three orders of magnitude of concentrations.

3.2.6 Normalization strategy

The *Human T_{Reg} Chip* provides rapid, parallel surveys of gene-expression patterns for hundreds of genes in a single assay. To analyse and handle the huge amount of generated data, an appropriate computational strategy had to be found. Software tools are rapidly evolving but no clear consensus exists about the "best" approach for gene expression data analyses. Indeed, it becomes increasingly clear that application of different techniques will allow different aspects of the data to be explored [6]. Consequently, choosing an appropriate algorithm for analysis was a crucial element of the experimental design.

To find this optimal normalization strategy, global approaches were tried as well as methods relying on housekeeping genes only, linear *versus* logarithmic and scaling

versus non-scaling algorithms were applied to the data set and normalization by mean and median values was compared. During this process, it became obvious that global normalization methods are not appropiate for the data, as they require equal overall signal intensities for all microarrays independently from the hybridized sample material. Thus, global normalization strategies can only be applied when the number of differentially expressed genes can be neglected compared to the total number of test genes of the microarray. This requirement cannot be fulfilled for customized approaches including the *Human T_{Reg} Chip* that was designed to point out differential gene expression between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Furthermore, only analyses of at least 20% of the whole transcriptome can guarantee equal signal intensity levels [188]. That is why normalization was performed by including just the housekeeping genes that are equally expressed by both analysed T cell subpopulations.

Boxplots are a good tool to illustrate how different normalization procedures affect data distribution. Figure 15 summarizes boxplots for all done RCC microarrays before and after data normalization. As demonstrated, the majority of values is located in a raw signal intensity range between 1 and 10^4 before normalization. Strikingly, raw data of the various microarrays clearly differ in their distribution pattern, especially in their median and mean values. Thereby, the mean is always higher than the median resulting from an overestimation of extremely big signal intensities when calculating the mean. That is why the median is more robust to outliers. To compensate for all these variances, normalization of raw data is indispensably required. Finally, the data was normalized similar to a proposal of MWG (for details see Materials and methods) by the following formula:

$$SI_{\text{norm}} = \frac{I_n - B_n}{e^{<ln(house)>}}$$

Where SI_{norm} is the normalized signal intensity, I_n is the signal intensity of gene n, B_n is the background intensity of gene n, and <ln house> is the median logarithmic signal intensity of all housekeeping genes. MWG's algorithm was improved by using the median instead of the mean to make the approach less susceptible to possible outliers. This data normalization procedure led to the most homogeneous data distribution pattern. As shown in Figure 15, most of the values are now located in a signal intensity range between 10^{-2} and 10^2 and additionally the medians became more similar. Extremely low values are evened out as demonstrated by the 1st percentile and the minimum, while differences of the maximum values seem to get a little bigger. Thus, the applied normalization strategy results in a transformation to smaller values, compensates for differences in data distribution parameters and symmetrically arranges the data around an arithmetic mean of the distribution located at $10^0 = 1$.

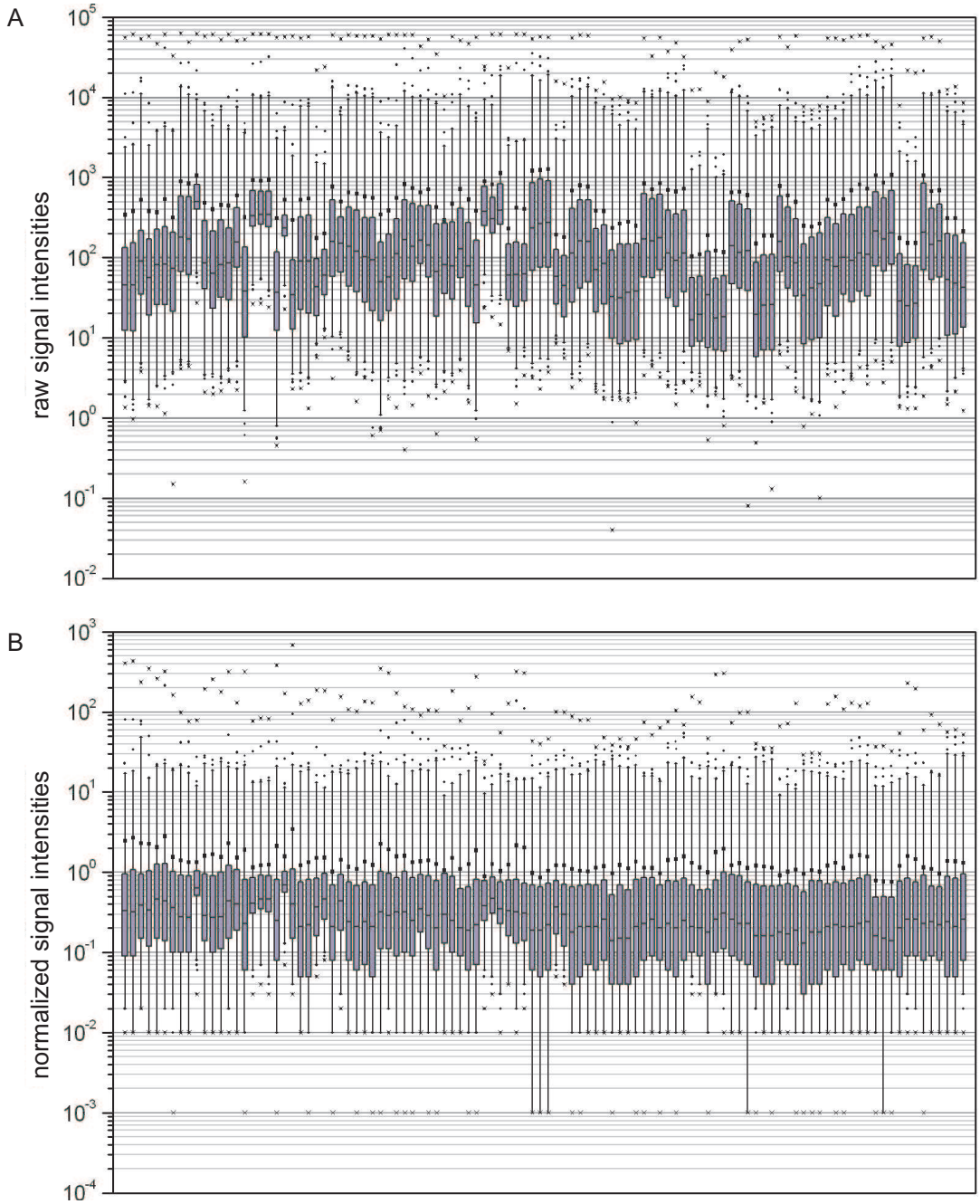

Figure 15: Boxplots of raw and normalized signal intensities of all test genes. Distribution of signal intensities for all done RCC microarray experiments before and after normalization is depicted. Vertical boxes contain 50% of all values falling between 25th and 75th percentile. The horizontal lines within the bars illustrate the median of the distribution and black squares (■) show its arithmetic mean. Horizontal lines extending from the boxes include 98% of all values (1st to 99th percentile), minimum and maximum value are represented by a cross (×) and outliers are depicted as black points (•). Adapted from [253]

3.3 Human regulatory T cells in health

3.3.1 Purity and phenotype of isolated T cell fractions

In order to obtain reliable and significant transcription profiles of human $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells, isolation of highly pure T cell subpopulations was indispensably required. Both T cell fractions were separated from PBMCs using MACS technology as described above. To assess purity of the isolated regulatory and naive T cell populations, cells were stained with CD4-FITC and CD25-PE antibodies and subsequently analysed by FACS. Thereby, expression of the surface molecules CD4 and CD25 was determined for living lymphocytes, as exemplified in Figure 16.

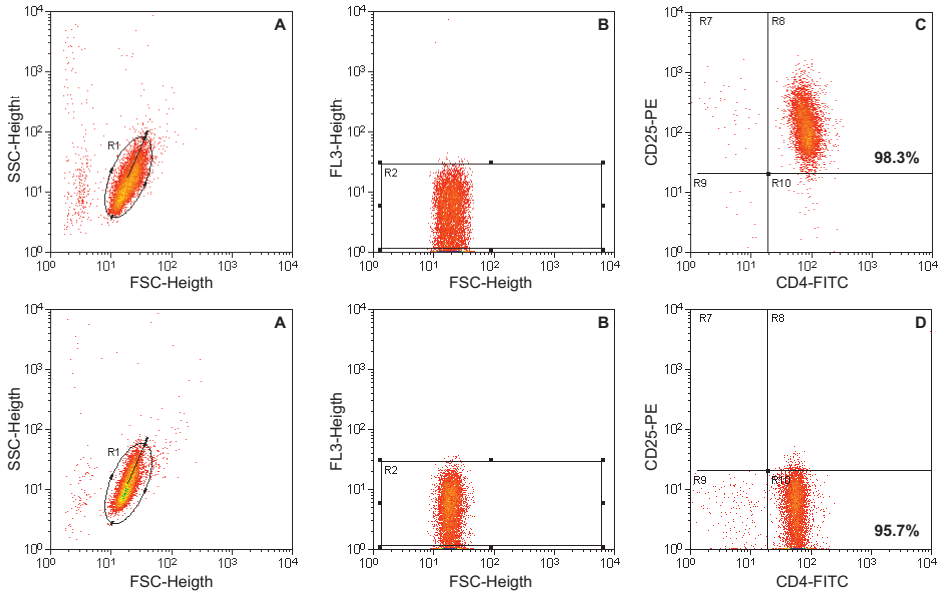

Figure 16: FACS strategy to assess purity of MACS separated human $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cell fractions.
A) Lymphocyte population in gate R1. B) Living PI^- lymphocytes (FL3 = PI = propidiumiodide) of gate R1 in gate R2. C) Isolated $CD4^+CD25^+$ T_{Reg} cells gated for R1 and R2. D) Separated $CD4^+CD25^-$ naive T cells gated for R1 and R2. Purity of the obtained T cell fractions were given as percentages of vital $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, respectively, based on the particular total cell fraction. Quadrants in C and D were set according to the unstained PBMCs.

To get a more comprehensive overview of the achieved purity of both separated T cell fractions, results of the FACS analyses for all donors are summarized in Table 1, while means for the different donor groups are shown in a bar chart in Figure 17. As demonstrated, the isolated human $CD4^+CD25^+$ T_{Reg} cells were normally clearly more than 90% pure, while the $CD4^+CD25^-$ naive T cell fraction achieved purities of more than 80% in average. Furthermore, it becomes obvious, that the obtained purities for both T cell subpopulations are similar between the different donor groups which is an essential requirement for the future intended comparison of T_{Reg} cells in health and disease.

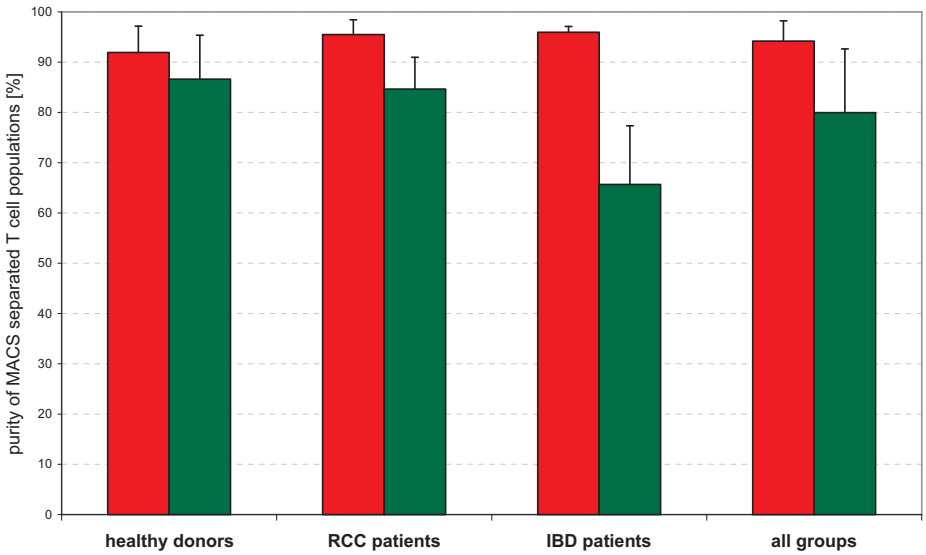

Figure 17: Mean purity of MACS separated T cell subpopulations.
Human T_{Reg} and naive T cells were isolated from PBMCs using MACS technology. Their purity was assessed by the FACS strategy explained in Figure 16 and expressed as means for either the $CD4^+CD25^+$ regulatory (red bars) or the $CD4^+CD25^-$ naive (green bars) T cell fraction, depicted separately for healthy donors, renal cell carcinoma (RCC) and inflammatory bowel disease (IBD) patients as well as for all groups together. Error bars indicate standard deviations.

Additionally, the phenotype of contaminating cells within the MACS purified $CD4^+$ $CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells was analysed by FACS. As depicted in Figure 18, remaining cells in both T cell subpopulations mainly represented $CD16^+/CD56^+$ natural killer cells and, at lower levels, $CD8^+$ T cells, $CD19^+$ B cells and $CD14^+$ monocytes.

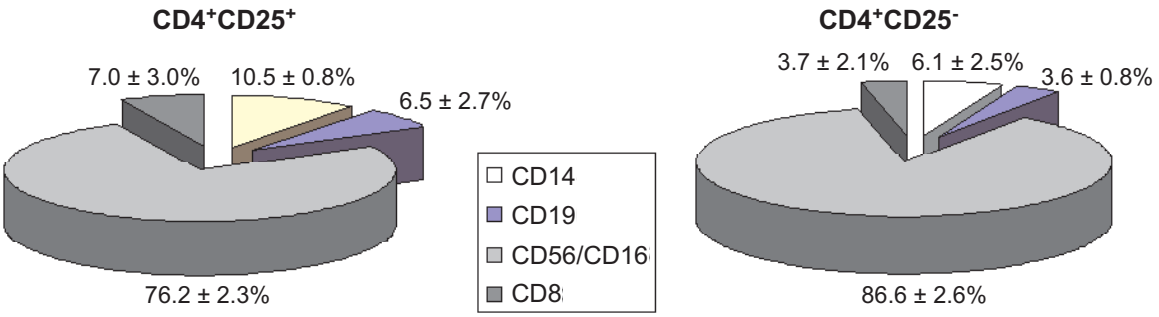

Figure 18: Phenotype of contaminating cells within MACS separated $CD4^+$ $CD25^+$ and $CD4^+CD25^-$ T cells.
Data was obtained from three healthy donors and re-analysed by FACS. The absolute number of contaminating cells was set to 100% and mean percentages of surface marker expression ± standard deviation are shown.

To confirm the regulatory phenotype of MACS purified $CD4^+CD25^+$ T cells, the fraction of FOXP3 expressing cells was estimated by intracellular FACS staining. Thereby, more than 80% of the isolated $CD4^+CD25^+$ T cells turned out to be FOXP3

positive [Figure 19A]. Additionally, suppressor function of MACS separated $CD4^+CD25^+$ T cells was assessed in proliferation assays that could confirm exhibition of regulatory T cell function *in vitro* [Figure 19]. As demonstrated, $CD4^+CD25^+$ T cells did almost not proliferate in response to allogeneic stimulation, while their naive counterparts showed strong proliferation under the same experimental conditions. Adding equal numbers of $CD4^+CD25^+$ T cells to the autologous $CD4^+CD25^-$ fraction resulted in a clear inhibition of their proliferation rate due to T_{Reg} cell suppressive activity.

Taken together, these results demonstrate that the applied separation strategy is able to enrich functional and phenotypic human $CD4^+CD25^+$ T_{Reg} cells.

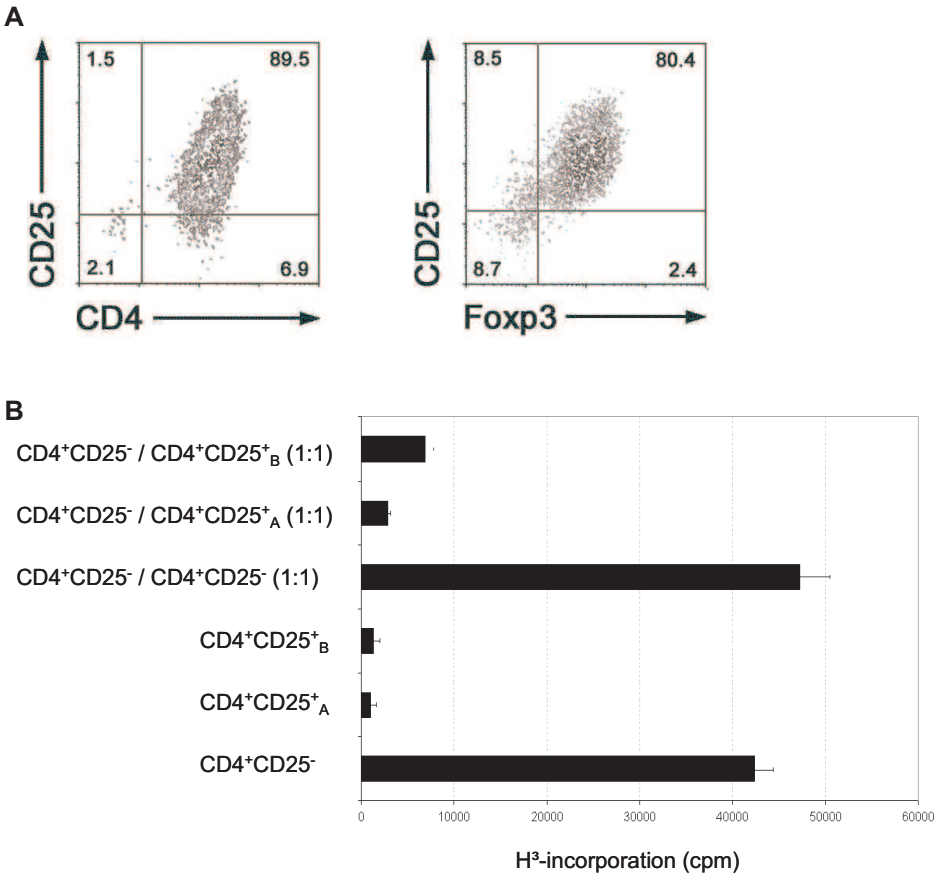

Figure 19: Regulatory phenotype and suppressor function of MACS purified human $CD4^+CD25^+$ T cells.
A MACS separated $CD4^+CD25^+$ T cells were analysed for cell surface CD4 and CD25 expression as well as intracellular FOXP3 expression. Percent of positive cells is indicated. Data shown is representative for three independent experiments. **B** To assess T_{Reg} cell proliferation and suppressive capacity, $CD4^+CD25^+$ and $CD4^+CD25^-$ derived T cells were stimulated with allogeneic EBV B cells alone or in the presence of equal numbers of indicated T cells. Proliferation was assessed at day 3, background proliferation was 891.3 ± 376.2 cpm.

3.3.2 Frequency of T_{Reg} cells in peripheral blood of healthy donors

In order to estimate prevalence of the isolated human $CD4^+CD25^+$ T_{Reg} cells, their frequencies in peripheral blood of 17 healthy donors was determined. Therefore, PBMCs were isolated, stained for CD4 and CD25 and subsequently analysed by FACS. $CD4^+CD25^{high}$ T cell numbers were evaluated as a percentage of total $CD4^+$ T cell population and resulted in an average frequency of 3.7% ± 1.9% in healthy volunteers [Figure 30].

3.3.3 Gene expression signatures of $CD4^+CD25^+$ T_{Reg} cells

One reason for the development of the *Human T_{Reg} Chip* was to provide a tool enabling improved molecular characterization of human T_{Reg} cells not by a single marker but by expression profiling. To obtain accurate and reliable individual transcription profiles, highly pure $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells from peripheral blood of 11 healthy donors were isolated using MACS technology [Table 1]. Each sample was measured in at least two independent microarray experiments. Following SAM analysis to identify genes with statistically significant changes in expression, 62 genes were found to be differentially expressed in regulatory compared to naive T cells in all healthy volunteers. Based on Gene Ontology and references in literature, genes were classified into functional categories, such as cytokines/chemokines and their receptors (12 genes), cell cycle and proliferation (11), apoptosis (7), signal transduction (9) and transcriptional regulation (10). A detailed description of these genes is summarized in Table 3. Among them, LGALS3, CCR7, IL2RA (CD25), CTLA4, TRAF1, SATB1 and GZMK were additionally found to be directly affected upon retroviral over-expression of FOXP3 in $CD4^+$ T_H cells [Figure 10].

gene symbol	gene name	disease association autoimmunity	cancer	transplantation	allergy
adhesion, cell growth and differentiation (4)					
NINJ2	ninjurin 2		HCC		
ACTN1	actinin, alpha 1	SLE, CHA	breast cancer		
NELL2	NEL-like 2		neuroblastoma, osteosarcoma, bladder, lung, pancreatic and prostate cancer		
ITGB2	integrin, beta 2	UC, MC, COPD, T2D, AS, LAD-1, RA, ALPS, SLE	prostate and bladder cancer, AML, CML	cGVHD, corneal, heart, kidney +BM allograft rejection	psoriasis, allergic lung inflammation, dermatitis, asthma
apoptosis (7)					
TIAF1	TGFB1-induced anti-apoptotic factor 1			chronic kidney and liver allograft rejection	
TP53INP1	tumor protein p53 inducible nuclear protein 1				
TRAF1 *	TNF receptor-associated factor 1		B-CLL		
LGALS1	galectin 1	JIA,RA, IBD	pancreatic adeno-carcinoma, colon, thyroid, bladder, ovary cancer, astrocytoma, melanoma	kidney allograft rejection, GVHD	
LGALS3 *	galectin 3	RA, JIA	HCC, NSCLC, melanoma, thyroid, breast and colon cancer		asthma
GZMA	granzyme A	T1D, RA, SLE, IBD	melanoma, leukemia	aGVHD, renal allograft rejection	CD, CMSE, HP
GZMK	granzyme K		CLL		
cell cycle and proliferation (11)					
PTTG1	pituitary tumor-transforming 1	diabetes	pituitary, adrenal, kidney, liver, ovarian and thyroid cancer, uterine leiomyoma		
TRIB1	tribbles homolog 1				
S100A10	S100 calcium binding protein A10		squamous NSCLC, ESCC, colorectal and gastric cancer, diffuse large B-cell carcinoma		
CEB1	hect domain and RLD 5				
SLAMF1	signaling lymphocytic activation molecule family member 1	SLE, X-linked XLP, RA, MS	Hodgkin's lymphoma		atopic dermatitis
S100A4	S100 calcium binding protein A4	RA	colon, breast and thyroid cancer, ESCC, pancreatic ductal adenocarcinoma	renal allograft rejection	
PIM1	pim-1 oncogene		prostate cancer, leukemia, diffuse large-cell lymphoma		
ID2	inhibitor of DNA binding 2	diabetes	neuroblastoma, HCC, pituitary adeno-carcinoma, APL, breast and prostate cancer		
FHIT	fragile hisT1Dine triad gene		esophageal, stomach, colon, lung, head and neck and cervical cancer, leukaemia, RCC		
RBMS1	RNA binding motif, single stranded interacting protein 1				
IFITM1	interferon induced transmembrane protein 1		gastric cancer, CML, ovarian carcinoma		
cytokines / chemokines and their receptors (12)					
IL2RA *	interleukin 2 receptor, alpha	T1D, profound cellular immunodefiency	T-cell lymphoma, breast cancer, T-LGLL, CLL	cutaneous GVHD	
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	MC, UC, MS, SLE	colorectal cancer, cholangiocarcinoma	corneal allograft rejection, aGVHD	
CCR5	chemokine (C-C motif) receptor 5	MS, Grave's disease, RA	RCC, HCC, colorectal cancer	aGVHD, renal and cardiac allograft rejection	asthma, HDM, atopic dermatitis
GPR2	chemokine (C-C motif) receptor 10	autoimmune skin diseases	CTCL, breast cancer metastasis		psoriasis, atopic or allergic-contact dermatitis
IL2RB	interleukin 2 receptor, beta	ITP, RA, osteoarthritis, hemolytic anemia	breast cancer		
G1P2	interferon, alpha-inducible protein		APL		
IL1RL2	interleukin 1 receptor-like 2				
IL7R *	interleukin 7 receptor	SCID, RA, SLE	leukemia, lymphoma, breast cancer, thymoma	HSCT	
CCR7 *	chemokine (C-C motif) receptor 7	diabetes, SLE, MS, RA, JIA	head and neck, prostate and gastric cancer, PCNSL, Hodgkin's lymphoma, melanoma	cGVHD, graft rejection	asthma, psoriasis
TNFSF5	CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)	HIGM1, Alzheimer disease, T1D, SLE, MS, AS, ITP	CLL, thyroid and lung cancer, Sezary syndrome	allograft rejection	asthma, airway hyperreactivity, psoriasis
CCL5	chemokine (C-C motif) ligand 5	EAT, MS, diabetes, SLE, RA	gastric cancer, mammary carcinoma, adult T-cell leukemia	Scl-GVHD	atopic dermatitis, asthma, HDM-induced chronic bronchitis, cat and mold allergens, psoriasis
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	MS, RA	HNSCC, NSCLC, breast, colon and ovarian cancer, non-Hodgkin's lymphoma, neurofibroma		
signal transduction (9)					
SDC4	syndecan 4		ovarian cancer, AML		
CTLA4 *	cytotoxic T-lymphocyte-associated protein 4	T1D, Grave's disease, SLE	B cell leukemia, Non-Hodgkin's lymphoma	allo- and xenograft rejection of cardiac, liver and pancreatic island transplantation	psoriasis, CD
TFRC	transferrin receptor		ESCC	allograft rejection	
AKAP2	A kinase (PRKA) anchor protein 2				
DGKA	diacylglycerol kinase, alpha				
PITPNC1	phosphaT1Dylinositol transfer protein, cytoplasmic 1				
TRGV9	T cell receptor gamma variable 9				
CD81	CD81 antigen		HCC, neuroblastoma		asthma
PECAM1	platelet/endothelial cell adhesion molecule	ITP, diabetes, AS, RA, CIA, MS	cervix carcinoma, prostate cancer	aGVHD	asthma, atopic dermatitis
transcriptional regulation (10)					
FOXP3	forkhead box P3	IPEX, T1D	head and neck and lung cancer, CLL, HCC	cGVHD	
GATA3	GATA binding protein 3	RA, HDR syndrome	Hodgkin's lymphoma, breast cancer, HCC		asthma
BHLHB2	basic helix-loop-helix domain containing, class B, 2	SLE	colon carcinoma, breast cancer		
SATB1 *	special AT-rich sequence binding protein 1				atopic dermatitis
STAT4	signal transducer and activator of transcription 4	MC, EAE, UC, diabetes, COPD, SLE, arthritis	lymphoma, CTCL	HSCT, aGVHD	HDM allergy, atopy
STAT6	signal transducer and activator of transcription 6	EAE, RA, autoimmune uveitis, diabetes	prostate and mammary carcinoma, fibro-sarcoma, mastocytoma, B-cell lymphoma	aGVHD	airway hyperrespon-siveness, asthma
MYC	v-myc myelocytomatosis viral oncogene homolog	diabetes, RA, SLE	APL, Burkitt lymphoma, plasmacytoma, breast, colorectal and prostate cancer		asthma, psoriasis
TCF7	transcription factor 7 (T-cell specific, HMG-box)	T1D	gut and mammary gland adenoma, colon cancer, CLL		Kawasaki disease, atopic dermatitis, asthma
XBP1	X-box binding protein 1	T2D, RA	breast and prostate cancer, myeloma, HCC		
CNOT2	CCR4-NOT transcription complex, subunit 2				
HLA molecules (3)					
HLA-DMA	major histocompatibility complex, class II, DM alpha	T1D, SLE, RA		kidney graft rejection	
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	RA, MS, sarcoidosis, Sjogren syndrome, Grave's disease, T1D		GVHD	asthma
HLA-DRB3	major histocompatibility complex, class II, DR beta 3	SLE, RA, MS, sarcoidosis, Sjogren syndrome, Grave's disease			
other immune-related genes (2)					
GBP2	guanylate binding protein 2, interferon-inducible		papillary thyroid carcinoma		
GBP5	guanylate binding protein 5		CTCL		
iron transport (a) and enzymes (b)					
SLC40A1 (a)	solute carrier family 40 (iron-regulated transporter), member 1				
SHMT2 (b)	serine hydroxymethyltransferase 2 (mitochondrial)				
unknown function (2)					
EPST11	epithelial stromal interaction 1		breast carcinoma		
NOSIP	nitric oxide synthase interacting protein				

Table 3: Genes differentially expressed between human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells from healthy donors.

JIA = juvenile idiopathic arthritis,RA = rheumatoid arthritis, CIA = collagen-induced arthritis, SLE = systemic lupus erythematosus, CHA = autoimmune chronic active hepatitis, UC = ulcerative colitis, MC = Morbus Crohn, COPD = chronic obstructive pulmonary disease, T2D = type II diabetes, T1D = type I diabetes, AS = atherosclerosis, LAD-1 = leukocyte adhesion deficiency-1, ALPS = autoimmune lymphoproliferative syndrome, IBD = inflammatory bowel disease, XLP = X-linked lymphoproliferative syndrome, MS = multiple sclerosis, ITP = idiopathic thrombocytopenic purpurea, SCID = severe combined immunodefiency, HIGM1 = hyper-IgM immunodefiency syndrome type I, ITP = immune thrombocytopenic purpura, EAT = experimental autoimmune thyroiditis, IPEX = immunodysregulation, polyendocrinopathy, and entheropathy, X-linked, EAE = experimental autoimmune encephalomyelitis, HCC = hepatocellular carcinoma, NSCLC = nonsmall lung cell cancer, AML = acute myelocytic leukaemia, CML = chronic myeloid leukaemia, (B)-CLL = (B-cell) chronic lymphatic leukaemia, ESCC = esophageal squamous cell carcinoma, APL = acute promyelocytic leukaemia, RCC = renal cell carcinoma, T-LGLL = T-cell large granular lymphocytic leukaemia, CTCL = cutaneous T-cell lymphoma, PCNSL = primary central nervous system lymphoma. HNSCC = head and neck squamous cell carcinoma, (a/c)GvHD = (acute/chronic) graft-*versus*-host disease, Scl-GvHD = sclerodermatous graft-*versus*-host disease, HSCT = hematopoietic stem cell transplantation, CD = celiac disease, CMSE = cows milk protein sensitive entheropathy, HP = hypersensitivity pneumotitis, HDM = house dust mite, TF = typhoid fever. *Genes that were additionally found to be differentially expressed upon retroviral over-expression of FOXP3 in $CD4^+CD25^-$ T cells. red = up-regulated, green = down-regulated genes

3.3.3.1 Hierarchical clustering analysis An unsupervised, two-dimensional hierarchical clustering analysis was performed by entering the generated data set into Genesis software, revealing the displayed gene expression profile of regulatory T cells [Figure 20]. Thereby, coexpressed genes and replicated experiments were arranged next to each other. Furthermore, the transcriptional pattern clearly separated $CD4^+CD25^+$ regulatory from $CD4^+CD25^-$ naive T cells and distinguished between 32 up- and 30 down-regulated genes.

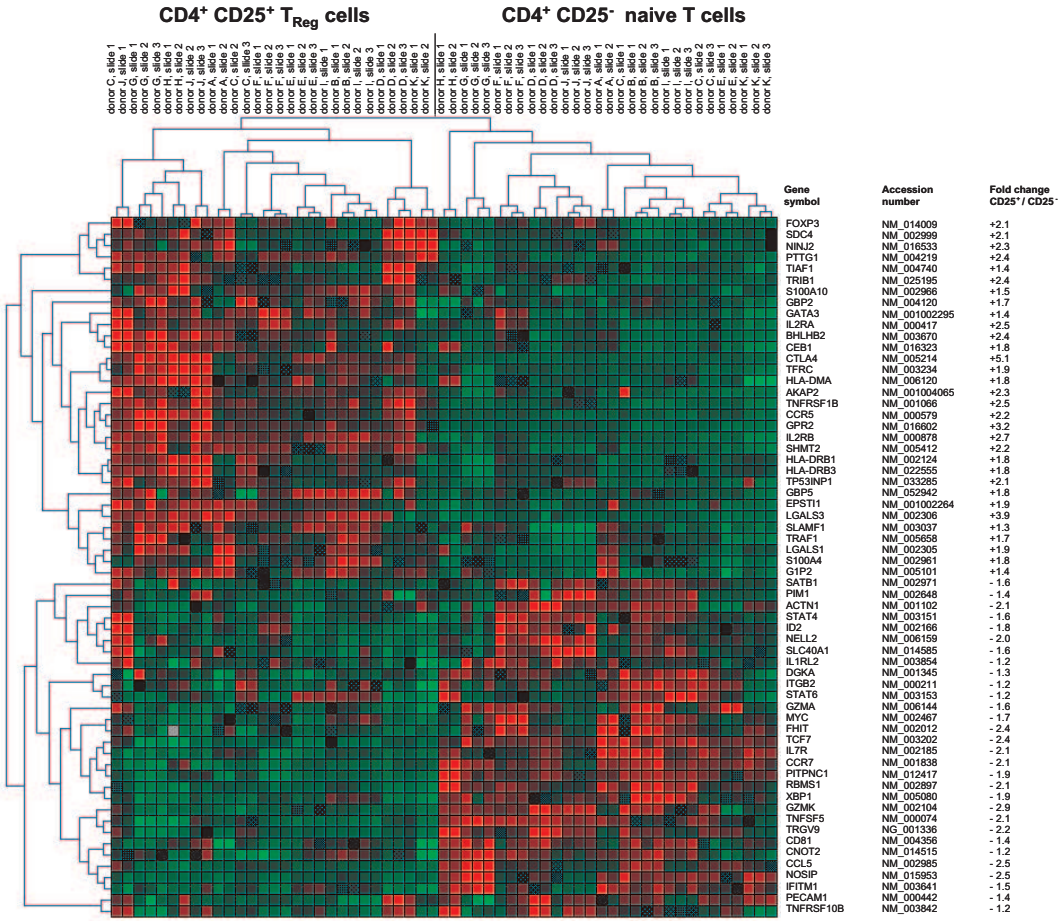

Figure 20: Transcriptional profiling of $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells.
To reveal molecular differences between regulatory and naive human T cells, differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, Statistical Analysis of Microarrays (SAM) was applied as a data mining tool to ascertain gene expression changes, disclosing 62 significantly altered genes between both T cell subpopulations (delta = 2.46, median FDR = 0.48). After entering the generated data set into Genesis software a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory from naive T cells consisting of 32 up-regulated and 30 down-regulated genes. Each row represents a gene probed on the *Human T_{Reg} Chip*; each column shows expression of the 62 genes measured for each individual in the study. Red indicates genes that are expressed at higher levels compared to the mean signal intensities of all experiments, while down-regulated genes are coloured in green, and black indicates signal intensities near the mean expression level.

3.3.3.2 Old friends and new players 21 out of these 62 genes have already been described in the literature as being associated with T_{Reg} cells of both mouse and human origin, e.g. FOXP3, CTLA4, IL2RA (CD25) and ITGB2 [Figure 21]. Recovery of these "old friends" confirmed the applied non-redundant microarray approach including the cell separation strategy. Among the 62 genes, eight that were previously only implicated in murine T_{Reg} cell biology, could also be detected to be differentially expressed in human T_{Reg} cells (LGALS1, IL7R, GATA3, SATB1, TNFRSF1B, TNFSF5, DGKA, CCR5). Altogether, 15 genes were identified that were similarly regulated in mouse and human. Those genes at the intersection of both organisms reflect high levels of interspecies conservation during the evolutionary process, thereby lending credibility to their important role in T_{Reg} cell development and function. In addition to FOXP3, CTLA4 and IL2RA, the chemokine receptor 7 (CCR7), the transferring receptor (TFRC) and integrin beta 2 (ITGB2) were also found in this intersection group between mouse and men. Furthermore, six genes previously associated with human T_{Reg} cells could be confirmed by these microarray experiments. Apart from the "old friends", 41 "new players" were identified that have not previously been mentioned in the context of human T_{Reg} cells at all.

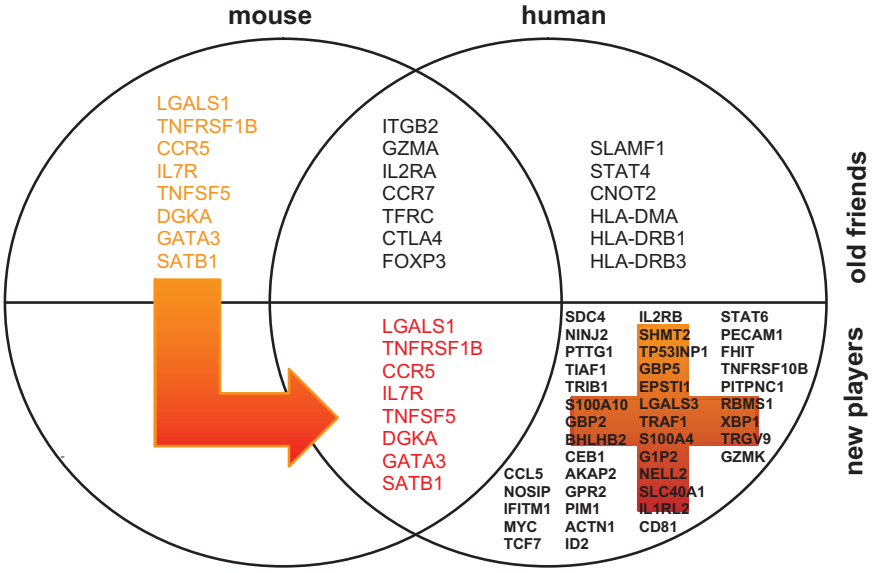

Figure 21: Old friends and new players.
Genes differentially expressed in regulatory and naive T cells as revealed by application of the *Human T_{Reg} Chip*. The upper half of the Venn diagram summarizes "old friends", i.e. T_{Reg} cell associated genes that have previously been described in literature for either mouse or human. The lower half of the chart illustrates the new situation by displaying all the "new players" of the T_{Reg} cell fingerprint. As demonstrated by the extended intersection, eight genes, formerly only reported to be implicated in mouse T_{Reg} cell immunology, were revealed to play an additional role in human T_{Reg} cell activity (red arrow). Furthermore, the obtained results broadened the knowledge about the transcriptional pattern characterizing human T_{Reg} cells by adding 41 new candidate genes (indicated by the red +).

3.3.3.3 Signaling modules in T_{Reg} cells revealed by pathway analysis To elucidate potential pathway modules implicated in T_{Reg} cell biology, PathwayAssist software was applied to the unique expression data set of human T_{Reg} cells from individual healthy donors. Mapping the 62 T_{Reg} cell specific genes yielded a network of 31 genes directly interacting with each other [Figure 22]. These 31 genes provided a comprehensive framework for further dissection into functional modules. These modules point to mechanisms controlling diverse cellular processes like survival/apoptosis, T cell receptor signaling/activation/proliferation and differentiation/maintenance of human T_{Reg} cells and are discussed in detail in chapter 4.2.2.2.

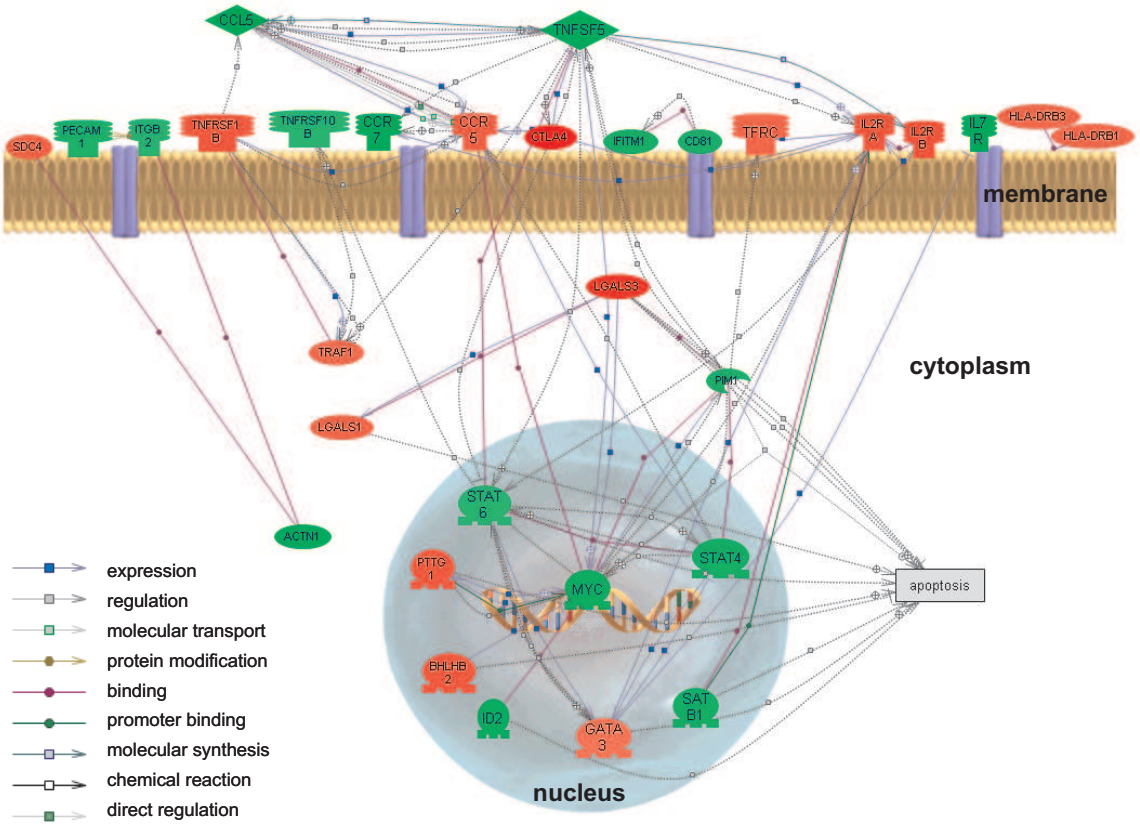

Figure 22: Pathway analysis of the 62 genes constituting the T_{Reg} fingerprint.
Genes significantly differentially expressed in regulatory *versus* naive human T cells were imported into PathwayAssist software and grouped according to the direct interaction algorithm of the pathway building tool. Unlinked nodes were excluded from this schematic representation, whereas the remaining 31 components were additionally assigned to their common cellular localization using the particular integrated layout option. Genes found to be up-regulated are shown in red, down-regulated nodes are coloured in green.

3.3.3.4 Implications in T_{Reg} cell mediated diseases Apart from the "old friends", the obtained T_{Reg} cell signature comprises 41 "new players" that have not

yet been described in T_{Reg} cells at all. As T_{Reg} cells have a far-reaching effect on our health by influencing the outcome of infection, autoimmunity, transplantation, cancer, and even allergy, it was studied whether these new candidates have been reported to participate in these processes. Interestingly, the vast majority of the genes identified in this study (51 out of 62) were implicated in at least one of these disease scenarios [Table 3]. To summarize, 32 genes were found to be involved in autoimmune disorders, 44 candidates to participate in tumor development, 20 genes influencing graft survival and 19 candidates to contribute to allergic disease pathogenesis. Chapter 4.2.2.3 provides a detailed discussion of the genes in these T_{Reg} cell mediated diseases.

3.3.4 More news about the phenotype of human T_{Reg} cells

3.3.4.1 Confirmation of microarray gene expression data To verify accuracy of the obtained microarray data in more detail, quantitative real-time RT-PCR was performed using the original samples. Referring to well-characterized T_{Reg} cell genes (FOXP3, CTLA4 and CCR7) the chosen approach could be confirmed [Figure 23A-C]. This gave greater credence and reliability to the numerous additional genes that have not yet been reported in (human) T_{Reg} cells. Five of these "new players" (LGALS3, CCR10, TNFRSF1B, CCR5, TRAF1) were selected and their T_{Reg} cell specific expression was approved by quantitative real-time RT-PCR [Figure 23D-H]. As depicted, PCR results generally correlated well with the differential gene expression data obtained by application of the *Human T_{Reg} Chip*. For a few donors variability in gene expression was observed between microarray and quantitative RT-PCR data, but the direction of change was consistent, lending confidence to the reliability of the *Human T_{Reg} Chip* results and the chosen cell separation strategy.

3.3.4.2 Protein expression by FACS analysis As post-transcriptional mechanisms can modify mRNA stability, it was assessed whether gene expression correlated on mRNA and protein level. Therefore, four interesting target proteins were selected, two of them are expressed at the surface (TNFRSF1B, CCR10) and two intracellularly (FOXP3, LGALS3). Expression of these proteins was determined by three-colour staining of isolated PBMCs and subsequent FACS analyses.

As shown in Figure 24, FOXP3 antibody staining revealed that almost all $CD4^+$ $CD25^{high}$ T_{Reg} cells ($84.3\% \pm 3.5\%$) but only very few $CD4^+CD25^-$ naive T cells ($3.7\% \pm 0.8\%$) expressed this protein intracellularly. This finding is thereby confirming the applied gating strategy as FOXP3 is generally accepted as the best marker for regulatory T cells available to date. Furthermore, it could be demonstrate that $CD4^+CD25^{high}$ T_{Reg} cells expressed significantly higher amounts of TNFRSF1B and LGALS3 protein than their naive counterparts as shown by the shifts in the histogram overlays [Figure 24A]. An average of $64.4\% \pm 4.9\%$ T_{Reg} cells *versus* $13.8\% \pm 3.4\%$ of $CD4^+CD25^-$ T cells showed TNFRSF1B protein expression on their surface, while $23.5\% \pm 7.0\%$ T_{Reg} cells compared to only $0.5\% \pm 0.06\%$ of $CD4^+CD25^-$ T cells expressed intracellular LGALS3 protein [Figure 24B].

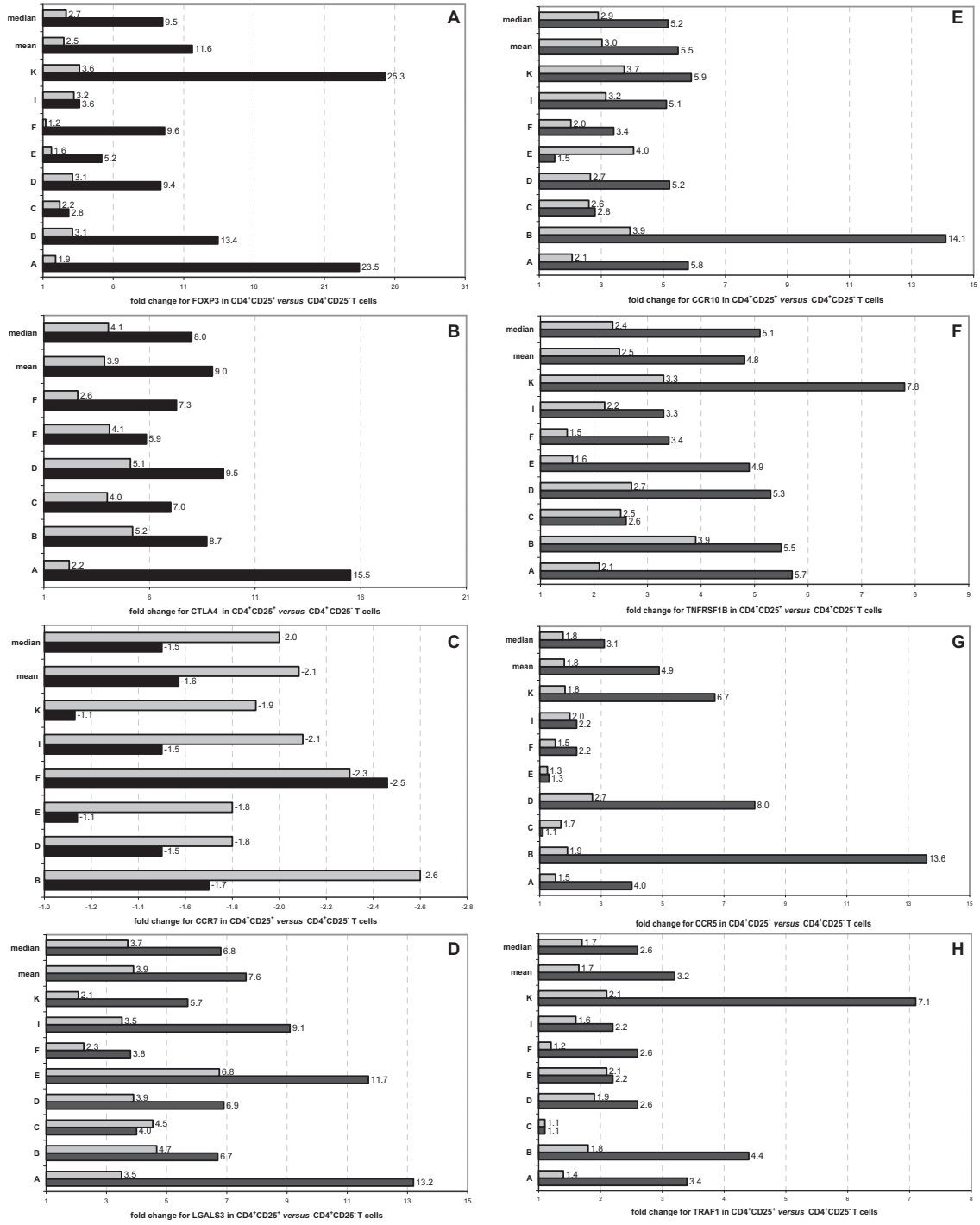

Figure 23: Old friends and new players: Confirmation of microarray results. Real-time RT-PCR was performed for FOXP3 (A), CTLA4 (B), CCR7 (C), LGALS3 (D), CCR10 (E), TNFRSF1B (F), CCR5 (G), TRAF1 (H) and RPS9 (data not shown) expression in MACS separated human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells. Following normalization to RPS9, relative mRNA amounts in $CD4^+CD25^+$ T_{Reg} cells were adjusted to corresponding expression levels in $CD4^+CD25^-$ naive T cells and expressed as fold changes. Real-time RT-PCR results, indicated by black bars, were compared to fold changes arising from the *Human T_{Reg} Chip* (represented by grey bars). The particular healthy donors were specified by letters according to Table 1. Furthermore, mean and median fold changes for all indicated volunteers were given for each gene.

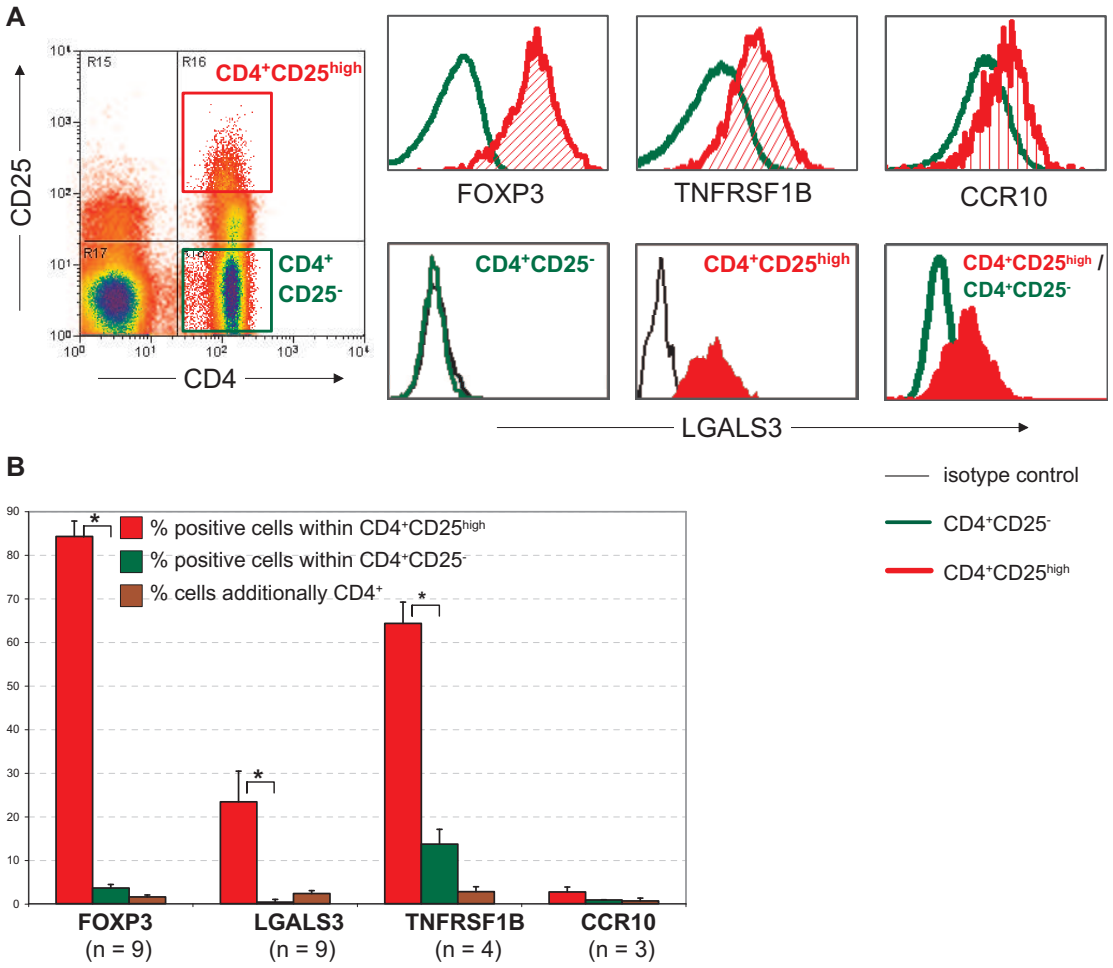

Figure 24: Protein expression by human $CD4^{+}CD25^{high}$ regulatory and $CD4^{+}CD25^{-}$ naive T cells.

PBMCs of healthy donors were isolated, stained for CD4, CD25 and a third protein-of-interest. **A** Gated $CD4^{+}CD25^{high}$ T_{Reg} (upper red panel) and $CD4^{+}CD25^{-}$ naive (lower green panel) T cells were analysed for either FOXP3, LGALS3, TNFRSF1B or CCR10 protein expression by FACS. Representative overlays for the particular protein expression in regulatory (red histograms) and naive (green histograms) T cells are shown. **B** Percentages of cells expressing the protein-of-interest in the different T cell subpopulations (red and green bars) as well as fraction of cells that were positive for CD4 and the target protein (brown bars) were summarized for all analysed donors as means ± standard deviation (n = number of healthy volunteers, $^{*}P < 7{\cdot}10^{-6}$).

In contrast to that, just a very low over-expression of CCR10 protein was found in $CD4^+CD25^{high}$ T_{Reg} cells reflected in the smallest shift in the histogram overlays among all candidate proteins. Furthermore, only a marginal fraction of T_{Reg} cells at all (2.8% ± 1.1%) expressed CCR10 at their surface. Taken together, significantly higher amounts of FOXP3, LGALS3 and TNFRSF1B protein expression could be confirmed in human $CD4^+CD25^{high}$ T_{Reg} cells compared to $CD4^+CD25^-$ T cells of healthy donors.

To get a better impression of the frequency of positive cells within the total lymphocyte population, the percentages of $CD4^+$ T cells expressing the particular protein-of-interest were additionally assessed. In average, 1.7% $CD4^+FOXP3^+$, 2.4% $CD4^+$ $LGALS3^+$, 2.9% $CD4^+TNFRSF1B^+$ and only 0.8% $CD4^+CCR10^+$ T cells were found in the living lymphocyte population [Figure 24B, brown bars]. These results reflect similar protein expression ranges of LGALS3 and TNFRSF1B, respectively, compared to FOXP3.

Comparing gene expression data obtained by microarray analyses and real-time RT-PCR to protein synthesis measured by FACS, could clearly demonstrate a strong correlation between mRNA and protein levels for FOXP3, LGALS3, TNFRSF1B and even CCR10 expressed as fold changes between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells [Figure 25]. Thereby, these findings again lend confidence to the microarray results.

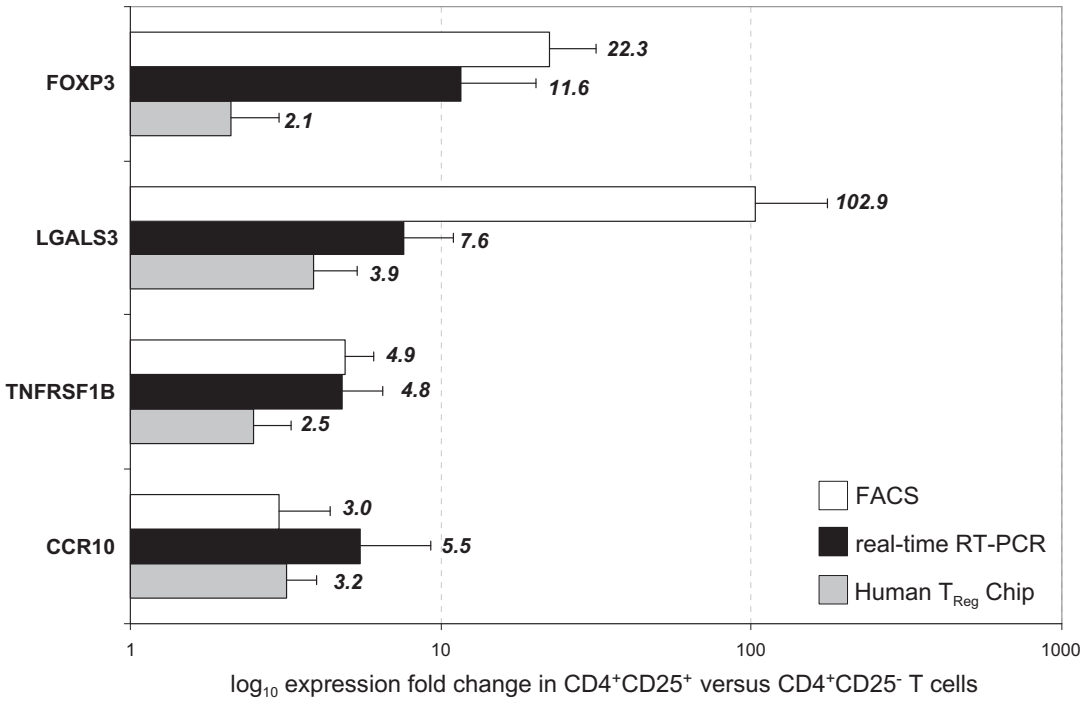

Figure 25: Correlation of gene and protein expression data.
Gene expression data obtained by *Human T_{Reg} Chip* analyses (n = 11) and real-time RT-PCR (n = 8) as well as protein expression data (number of analysed healthy donors as given in Figure 24B) revealed by FACS analyses are presented as logarithmic fold changes in MACS separated healthy human $CD4^+CD25^+$ *versus* $CD4^+CD25^-$ T cells. Values are given as means of all analysed donors, error bars indicate standard deviation.

Taken together, significantly higher gene and protein expression of FOXP3 could be confirmed in $CD4^+CD25^{high}$ T_{Reg} cells compared to $CD4^+CD25^-$ T cells. Furthermore, correlating up-regulation of LGALS3 and TNFRSF1B expression on gene and protein level by healthy human T_{Reg} cells has been proven. Both new candidate marker molecules were demonstrated to be expressed in similar amounts as FOXP3.

3.3.4.3 Coexpression of LGALS3, TNFRSF1B and FOXP3 protein To date, FOXP3 is widely accepted as the most suitable human T_{Reg} cell marker available. To further examine qualification of LGALS3 and TNFRSF1B as possible new candidate markers, coexpression of both proteins with FOXP3 was assessed. Three-colour staining with subsequent FACS analysis of isolated PBMCs from healthy donors revealed that double positive $CD4^+$ T cell fractions indeed exist. As illustrated in Figure 26, approximately 1.5% of gated $CD4^+$ T cells coexpress LGALS3 and FOXP3 intracellularly, while 0.5% coexpress TNFRSF1B on the surface and intracellular FOXP3. Furthermore, it becomes obvious, that there is an even more extended population of $CD4^+$ T cells expressing just the protein-of-interest, but not FOXP3. In average, 7.5% of analysed cells are $CD4^+$ $LGALS3^+$ and 5.8% are $CD4^+$ $TNFRSF1B^+$, respectively, but show no detectable FOXP3 protein expression. Conversely, a considerable part of the $CD4^+$ $FOXP3^+$ T cell population is negative for LGALS3 (2.4% ± 0.3%) or TNFRSF1B (4.8% ± 0.9%) protein expression. Whether these different T cell subpopulations differ in their regulatory phenotype and suppressive capacity remains to be elucidated.

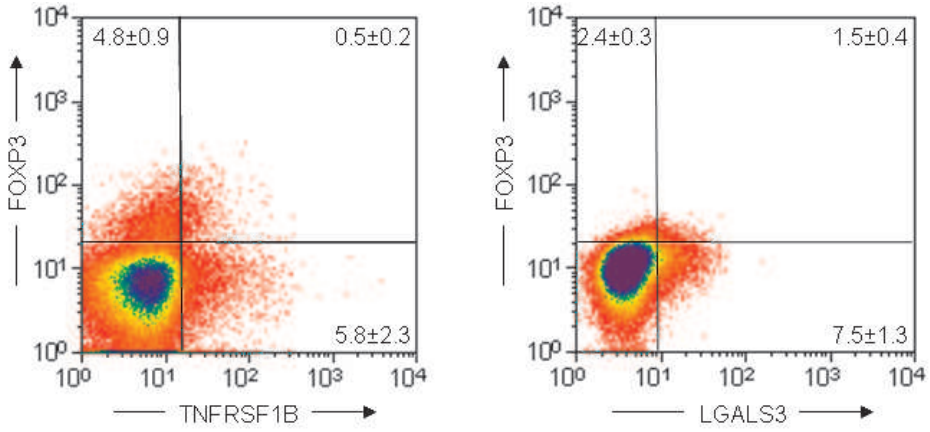

Figure 26: LGALS3 as well as TNFRSF1B are coexpressed with FOXP3 protein.
PBMCs were isolated by MACS from nine healthy volunteers, stained with CD4-PE, FOXP3-APC and either LGALS3-FITC or TNFRSF1B-FITC. For FACS analyses cells were gated for living $CD4^+$ lymphocytes. The depicted dot plots are representative for all nine donors analysed. Indicated percentages represent positive cells in the particular quadrant and are given as means ± standard deviation.

3.3.4.4 Putative new marker molecules Characterization, enumeration and manipulation of human T_{Reg} cells would be greatly facilitated by the identification of a specific marker molecule capable to distinguish them from naive and recently activated T cells. Optimally this marker should be a cell surface receptor that is highly expressed exclusively in T_{Reg} cells independently from their activation status. In contrast to CD25, CTLA4 and GITR (which are examples of surface proteins commonly used to identify T_{Reg} cells), putative new markers should additionally not be induced upon activation of $CD4^+CD25^-$ T cells [15].

Comparative gene expression analyses of human *ex vivo* $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells applying the *Human T_{Reg} Chip* revealed several new candidate genes that could potentially function as marker molecules [Figure 21]. Differential gene expression of selected candidates, namely of LGALS3, CCR10, CCR5, TNFRSF1B and TRAF1 was confirmed by real-time RT-PCR [Figure 23]. Finally, FACS analyses verified up-regulation of LGALS3, TNFRSF1B and - to a lesser extent - CCR10 proteins in these *ex vivo* human T_{Reg} cells compared to their naive counterpart [Figure 24].

Next, the question was addressed whether these genes/proteins are constitutively expressed on $CD4^+CD25^+$ T cells even after *in vitro* activation. Furthermore, their expression status in recently activated $CD4^+CD25^-$ T cells was of special interest. To clarify these points, human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells were isolated from the peripheral blood of healthy donors using MACS. Purity of both cell fractions was ensured by FACS analysis as described before. Afterwards, both T cell subpopulations were cultured independently from each other in appropriate medium and were activated by plate-bound anti-CD3 in a final concentration of 1 μg/ml and 100 U IL2. As much more $CD4^+CD25^-$ T cells than T_{Reg} cells can be isolated, controls of naive T cells activated with anti-CD3 only (1 μg/ml) and such stimulated just with IL2 (100 U) were added to the experiment. Some of the cells were harvested at day 0 and day 3 for subsequent real-time RT-PCR, while the rest of the cells was analysed for their protein expression by FACS at day 0 and 5.

Figure 27 summarizes results of the real-time RT-PCR analyses comparing gene expression of LGALS3, FOXP3, TNFRSF1B, TRAF1, CCR10 and CCR5 in *ex vivo* isolated to recently *in vitro* activated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. As illustrated, LGALS3, TNFRSF1B, TRAF1 and CCR10 are constitutively over-expressed by T_{Reg} cells independently from their activation status as the fold changes to their naive counterparts remain constantly high after *in vitro* stimulation with anti-CD3 and IL2. Expression status of these four candidates thereby follows the expression pattern of FOXP3, which is generally accepted as the most suitable T_{Reg} marker currently available. In contrast to that, CCR5 expression clearly decreases after *in vitro* activation of human T_{Reg} cells, but is still higher than in *ex vivo* $CD4^+CD25^-$ T cells. Extending these analyses to activated $CD4^+CD25^-$ T cells revealed that CCR10 and CCR5 expression is strongly down-regulated. TNFRSF1B expression is slightly higher than in non-activated *ex vivo* $CD4^+CD25^-$ T cells, but still lower than in activated and *ex*

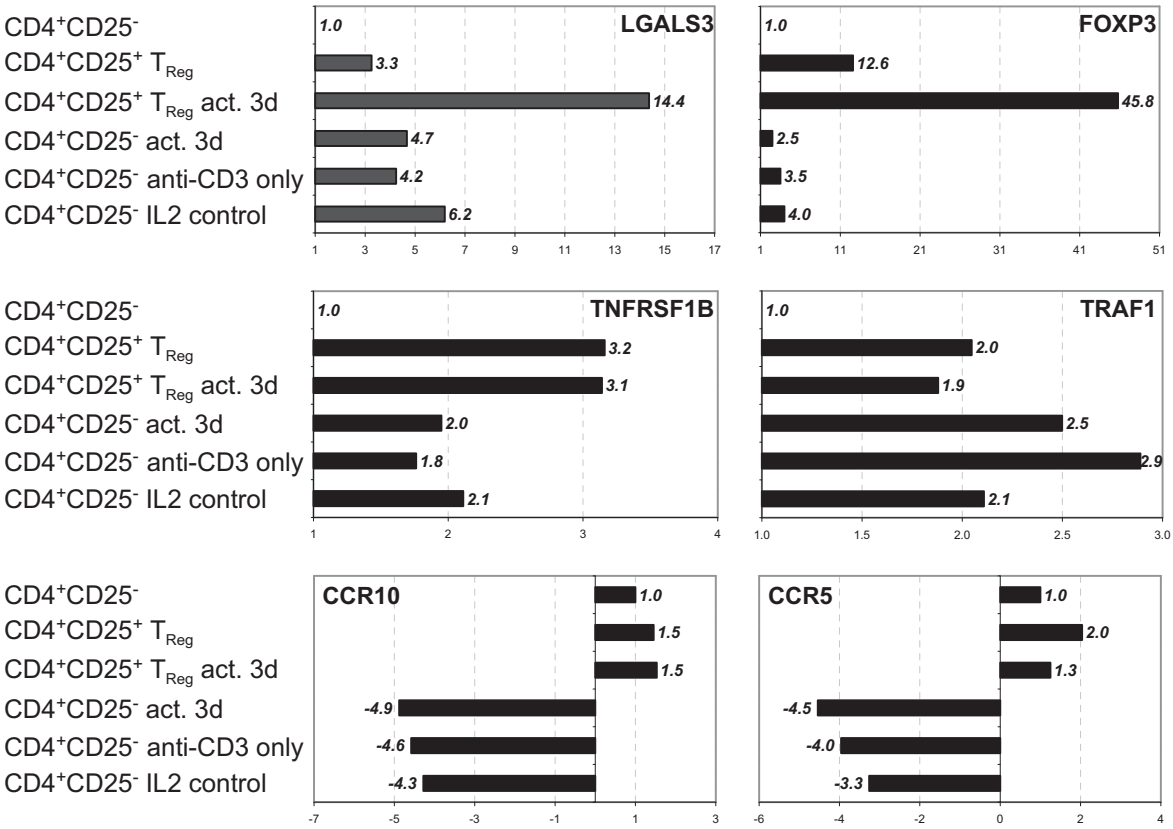

Figure 27: Gene expression of LGALS3, FOXP3, TNFRSF1B, TRAF1, CCR10 and CCR5 in naive, activated and regulatory human T cells as assessed by real-time RT-PCR.
PBMCs were isolated from peripheral blood of healthy human donors and separated by MACS into $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells. A part of these cells were activated with 1 μg/ml anti-CD3 and 100 U IL2 *in vitro*. As controls, $CD4^+CD25^-$ T cells stimulated either with anti-CD3 or IL2 alone were included. Total RNA was prepared after three days, reverse transcribed and relative mRNA expression levels of LGALS3, FOXP3, TNFRSF1B, TRAF1, CCR10, CCR5 and RPS9 (as an internal control) were analysed in duplicate real-time RT-PCR assays. Relative mRNA amounts were normalized to expression levels in $CD4^+CD25^-$ T cells (fold change = 1). Mean fold changes (n = 3) are illustrated.

vivo T_{Reg} cells, thereby following expression behaviour of FOXP3. LGALS3 expression is clearly up-regulated upon activation, thereby even exceeding expression levels in *ex vivo* T_{Reg} cells, but is still significantly lower than in activated T_{Reg} cells. In contrast to that, TRAF1 expression is strongly up-regulated in activated $CD4^+CD25^-$ T cells exceeding expression levels in all other cell populations analysed. This expression status clearly disqualifies TRAF1 from being a putative new marker of human T_{Reg} cells as a discrimination to recently activated T cells is not possible. Altogether, the same trends in results were obtained when stimulating the $CD4^+CD25^-$ T cells with either anti-CD3 or IL2 only.

Finally, correlation of gene and protein expression was verified for TNFRSF1B, LGALS3 and CCR10 in *ex vivo* compared to *in vitro* activated (5 days) $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells by FACS analysis. As illustrated in Figure 28, TNFRSF1B expression remained clearly up-regulated on $CD4^+CD25^+$ T_{Reg} cells upon 5 days of activation compared to *in vitro* activated $CD4^+CD25^-$ T cells.

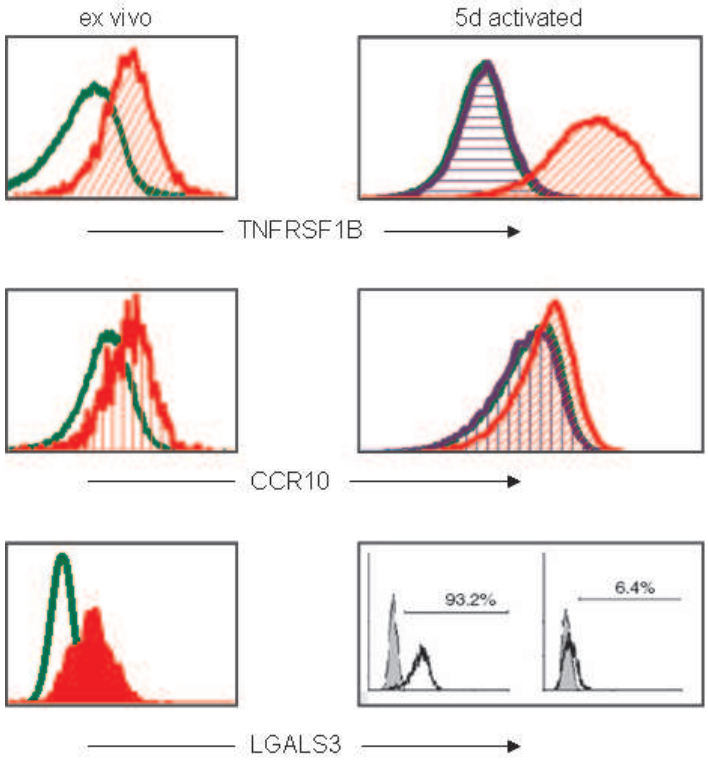

Figure 28: TNFRSF1B, LGALS3 and CCR10 expression in *ex vivo* and activated $CD4^+CD25^+$ T_{Reg} *versus* $CD4^+CD25^-$ T cells.
PBMCs were isolated from peripheral blood of healthy human donors and separated by MACS into $CD4^+CD25^+$ T_{Reg} (red histograms) and $CD4^+CD25^-$ T cells (green histograms). A part of these cells were activated with anti-CD3 (1μg/ml) plus IL2 (100 U) *in vitro*. As a control, some $CD4^+CD25^-$ T cells were stimulated with anti-CD3 only (blue histograms). After 5 days cells were harvested, stained for CD4, CD25 as well as for TNFRSF1B, LGALS3 and CCR10, respectively, and were subsequently analysed by FACS.

97% of activated T_{Reg} but only 16% and 12%, respectively, of anti-CD3 and anti-CD3 + IL2 activated $CD4^+CD25^-$ T cells were revealed to express TNFRSF1B at their surface. Similarly, high sustained levels of LGALS3 protein expression were only detected in activated T_{Reg} (93.2%) and almost no induction could be observed in activated $CD4^+CD25^-$ (6.4%) T cells. So, the shift in TNFRSF1B and LGALS3 expression between the subpopulations is even higher than that observed for freshly isolated (*ex vivo*) cells. Taken together, the gene and protein expression data for TNFRSF1B and LGALS3 on *ex vivo* and recently activated $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells, respectively, support the role of both molecules as new putative human T_{Reg} cell marker

molecules. In contrast to that, the shift for CCR10 protein expression after activation is still as small as for the freshly isolated T_{Reg} *versus* naive T cells not reflecting the gene expression data obtained by real-time RT-PCR. Since only a very small fraction expressed CCR10 at the surface and the over-expression after activation stayed low, CCR10 cannot further be considered as a marker molecule for human T_{Reg} cells.

3.3.4.5 Susceptibility to apoptosis As discussed later in more detail in chapter 4.2.2.2.1, the performed microarray analyses revealed several genes participating in apoptosis control. Using real-time RT-PCR [Figure 23] as well as flow cytometry [Figure 24], up-regulation of two anti-apoptotic genes / proteins, namely LGALS3 and TNFRSF1B, could be confirmed in human $CD4^+CD25^{high}$ T_{Reg} compared to naive $CD4^+CD25^-$ T cells. To elucidate whether both T cell subpopulations consequently differ in their apoptotic susceptibility, a combined Annexin V and propidium iodide (PI) staining of isolated PBMCs with subsequent FACS analysis was carried out [Figure 29]. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein with a high affinity for phosphatidylserine, a membrane phospholipid that is translocated from the inner to the outer leaflet in early stages of apoptosis. As PI enables staining of dead cells, Annexin V^- PI^- viable cells can be easily distinguished from Annexin V^+ PI^- apoptotic and Annexin V^+ PI^+ dead cells. Gating for $CD4^+CD25^{high}$ T_{Reg} and naive $CD4^+CD25^-$ T cells indeed revealed that regulatory T cells are less susceptible to apoptosis than their naive counterparts. As illustrated, a fraction of 2.6% ± 1.1% T_{Reg} *versus* 5.9% ± 1.0% naive T cells was demonstrated to undergo apoptosis. This difference in apoptotic behaviour was found to be highly significant ($P < 10^{-4}$).

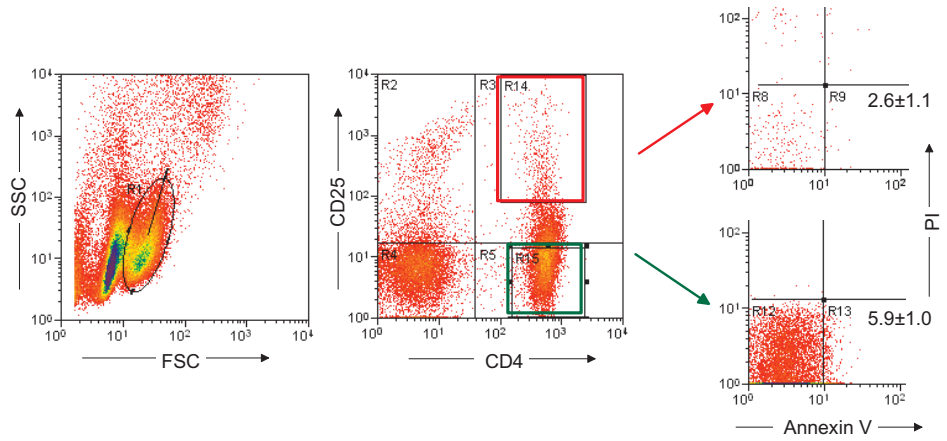

Figure 29: Human $CD4^+CD25^{high}$ T_{Reg} cells are less susceptible to apoptosis than $CD4^+CD25^-$ T cells.
PBMCs from seven healthy donors were isolated and stained for CD4, CD25 as well as with Annexin V and propidium iodide (PI). These cells were analysed by FACS, gated for lymphocyte population first (left panel) and afterwards for regulatory and naive T cells, respectively (middle panel). Percentages of apoptotic cells, which are Annexin V^+ but PI^- are given as means ± standard deviation (right panels).

3.4 Human regulatory T cells in RCC

3.4.1 Frequency of T_{Reg} cells in peripheral blood of RCC patients

As increased populations of T_{Reg} cells are thought to contribute to impaired anti-tumor immune responses in various human malignancies, T_{Reg} cell frequencies were investigated in peripheral blood of RCC patients and healthy donors. Therefore, $CD4^+CD25^{high}$ T cell numbers were evaluated by FACS as a percentage of the total $CD4^+$ T cell population using the gating strategy shown in Figure 30A. Representative dot plots of RCC patients and healthy volunteers are depicted there, while cumulative data are presented in a bar chart in Figure 30B.

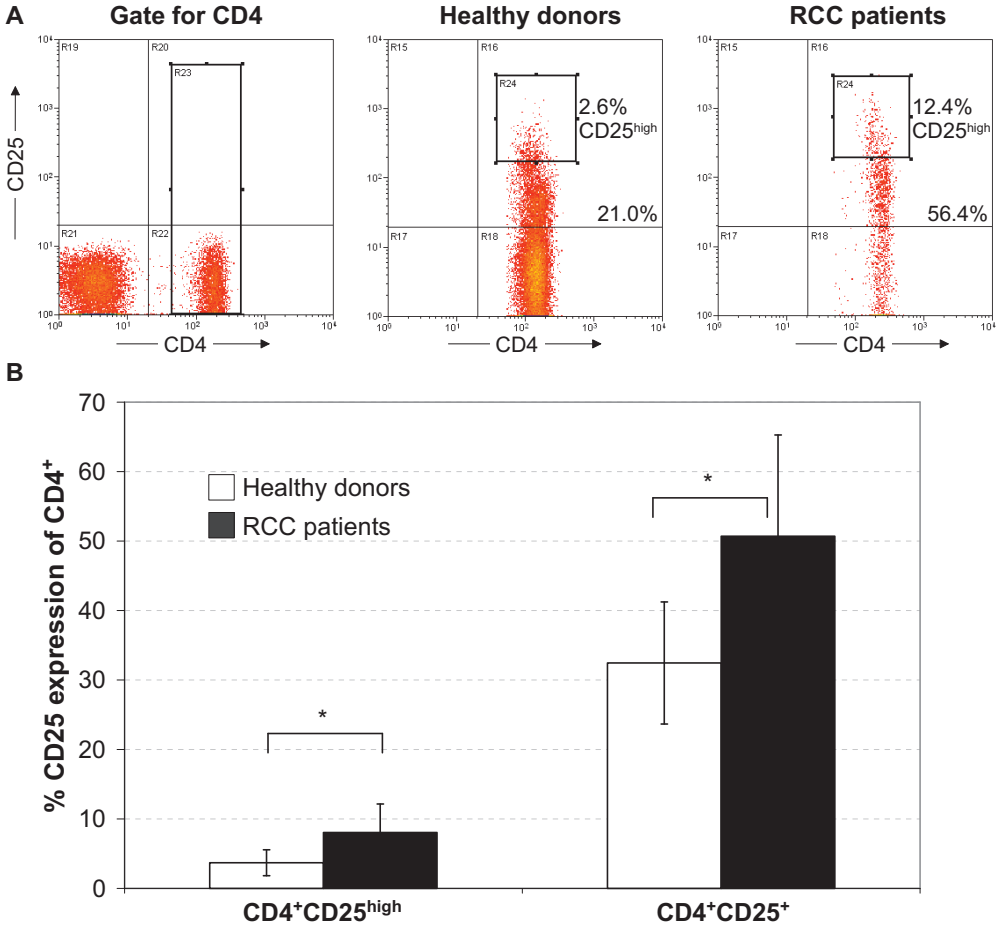

Figure 30: T_{Reg} cell frequency in health and RCC.
A Flow cytometric analysis of $CD4^+CD25^{high}$ T_{Reg} and $CD4^+CD25^-$ naive T cells in healthy donors and RCC patients. PBMCs were stained with anti-CD4-FITC and anti-CD25-PE. T_{Reg} cell frequency was determined as the percentage of brighter $CD25^+$cells on gated $CD4^+$ T cell population. Representative FACS results of a healthy donor and an RCC patient are shown. **B** Comparison of $CD4^+CD25^{high}$ T_{Reg} and $CD4^+CD25^+$ T cells between healthy individuals and RCC patients. Percentages of $CD4^+CD25^{high}$ and $CD4^+CD25^+$ T cells in the peripheral blood of 17 healthy donors (□) and 12 RCC patients (■) are shown. Values are given as means ± standard deviation. * $P < 0.004$

As demonstrated, the prevalence of $CD4^+CD25^{high}$ T cells in RCC patients (8.0% ± 4.1%, n = 12) was significantly higher than that in healthy individuals (3.7% ± 1.9%, n = 17, $P < 0.004$). Furthermore, elevated frequencies of $CD4^+CD25^{high}$ T cells in RCC patients were accompanied by an increase of total $CD4^+CD25^+$ T cell population, incorporating $CD25^{low}$ to $CD25^{dim}$ T cells. In RCC patients, 50.7% ± 14.6% of $CD4^+CD25^+$ T cells compared to 32.5% ± 8.8% in healthy individuals ($P < 0.002$, Figure 30B) were observed. In contrast to that, no significant differences in frequencies of $CD4^+CD25^{high}$ and $CD4^+CD25^+$ populations could be detected between localized (n = 7) and metastatic (n = 5) RCC (data not shown).

3.4.2 Gene expression signatures of $CD4^+CD25^+$ T_{Reg} cells in RCC

Apart from elevated T_{Reg} cell frequencies, exaggerated suppressive activity or increased homing to tumor sites can be supposed to contribute to tumor immune escape. To identify genes able to induce these characteristics in T_{Reg} cells from RCC patients, microarray analyses were performed applying the *Human T_{Reg} Chip*. Therefore, $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells were separated by MACS from six patients with localized and five patients with metastatic growth of RCC and processed as described before. All RCC patients' characteristics were summarized in Table 1. Altogether, 62 microarray experiments were carried out; 31 slides were hybridized with $CD4^+CD25^+$ and another 31 with $CD4^+CD25^-$ T cell-derived cRNA, respectively.

3.4.2.1 Regulatory *versus* naive T cells in RCC To identify genes significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells in RCC, microarray data was normalized and subjected to SAM and cluster analysis as previously described. The revealed transcriptional pattern arranged coexpressed genes and replicated experiments next to each other and clearly separated $CD4^+CD25^+$ T_{Reg} from $CD4^+CD25^-$ T cells. The gene expression signature of regulatory and naive T cells in RCC turned out to consist of 81 altered genes, which were composed of 49 up-regulated and 32 down-regulated candidates [Figure 31]. Expectedly, prominent ones were found among them, such as FOXP3, CTLA4 and IL2RA, but also numerous genes not previously associated with T_{Reg} cells at all were included (e.g. CCR8, CEB1, CDC25B).

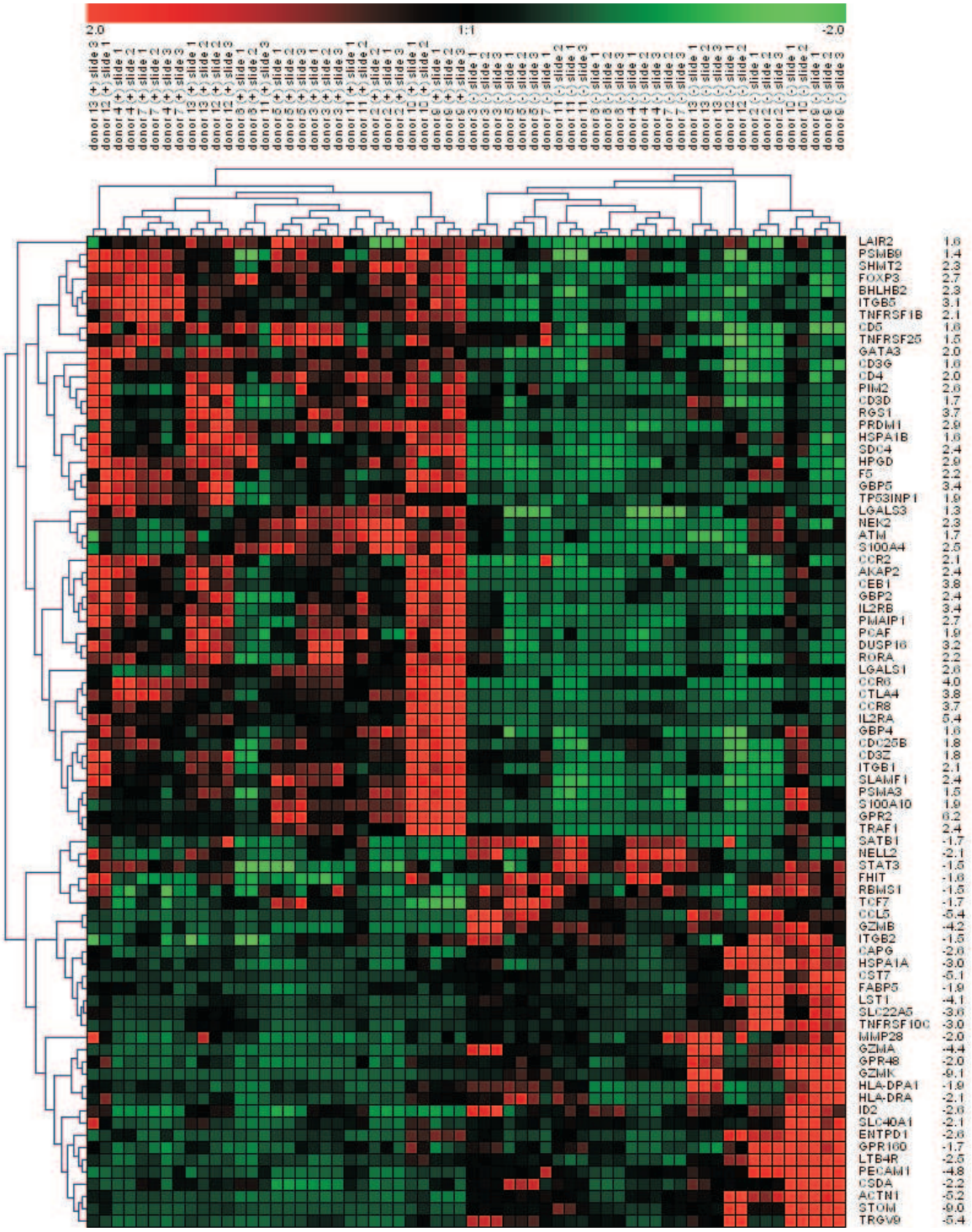

Figure 31: Transcriptional profiling of $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells from RCC patients.
To reveal molecular differences between regulatory and naive human T cells in RCC patients, differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, SAM was applied as a data mining tool to ascertain gene expression changes, disclosing 81 significantly altered genes between both T cell subpopulations (delta = 2.83, median FDR = 0.53). After entering the generated data set into Genesis software, a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory from naive T cells in RCC consisting of 49 up-regulated and 32 down-regulated genes. Each row represents a gene probed on the *Human T_{Reg} Chip*; each column shows expression of the 81 genes measured for each individual in the study. Red indicates genes that are expressed at higher levels compared to the mean signal intensities of all experiments, while down-regulated genes are coloured in green and black indicates signal intensities near the mean expression level.

3.4.2.2 Comparison to healthy donors As enhanced survival and potentially increased regulatory properties of T_{Reg} cells in RCC would be reflected in the revealed gene expression profile, it was compared to the transcriptional pattern obtained from T_{Reg} and naive T cells of healthy donors [Figure 20] which can be interpreted as a reference fingerprint. Although no genes with opposed regulations could be revealed between both donor groups, numerous genes could be detected that were just differentially expressed between T_{Reg} and naive T cells of either healthy donors (n = 27) or RCC patients (n = 46, [Figure 32]). Additional 35 genes were included in the expression profiles from T_{Reg} and naive T cells of both donor groups, but generally showed stronger regulation in RCC.

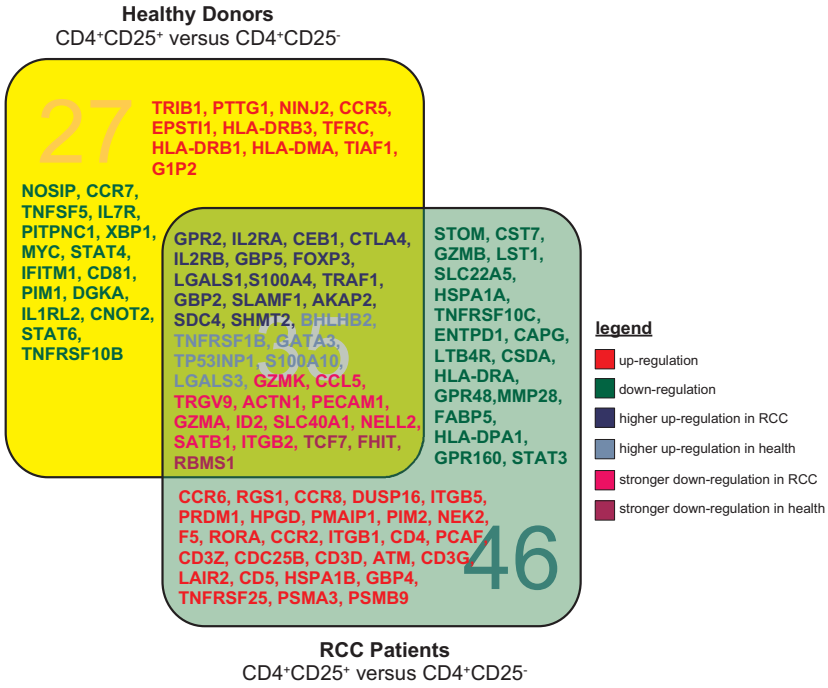

Figure 32: Similarities and differences in the gene expression signatures of T_{Reg} cells in RCC and health.
Genes significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells from 11 RCC patients and 11 healthy volunteers, respectively, were summarized in a Venn diagram. As depicted by the intersection, 35 genes were contained in the expression profiles of both donor groups, but generally showed stronger regulation in RCC. Additional 27 and 46 genes, respectively, were found to be just differentially expressed between T_{Reg} and naive T cells of either healthy donors or RCC patients. Genes were arranged according to their decreasing fold change between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells.

To reveal significant differences in the gene expression profiles of $CD4^+CD25^+$ T_{Reg} from healthy volunteers and RCC patients, microarray data of the same cell type but of different donor origin had to be compared. As illustrated in Figure 33, SAM and hierarchical clustering analysis identified 121 genes differentially expressed in T_{Reg} cells of both donor groups, composed of 71 up-regulated and 64 down-regulated genes when comparing RCC to health.

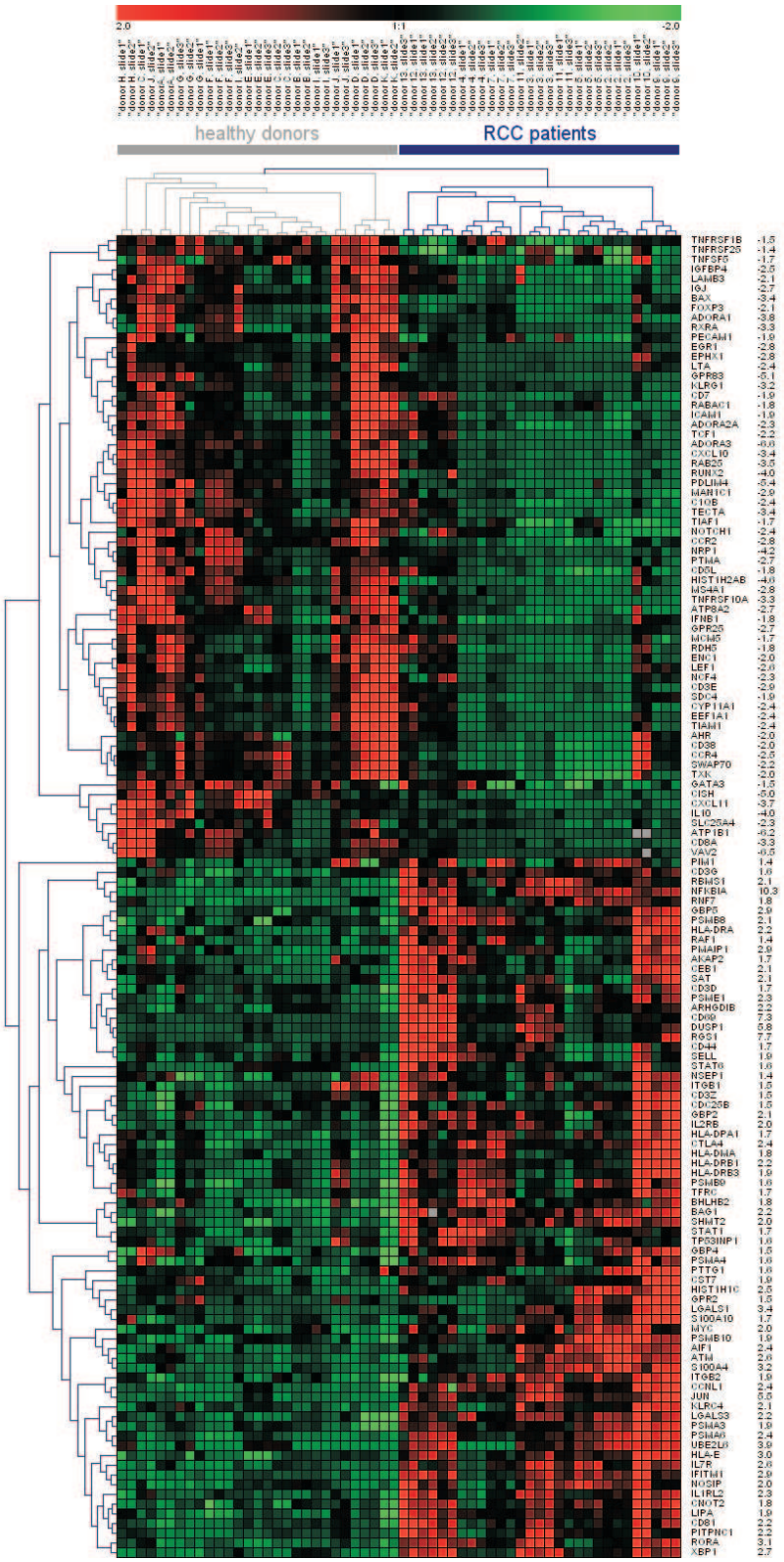

Figure 33: Expression profiling of $CD4^+CD25^+$ T_{Reg} from RCC patients and healthy donors.
To reveal molecular differences between regulatory T cells in RCC patients compared to healthy donors, differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, SAM was applied as a data mining tool to ascertain gene expression changes, disclosing 121 significantly altered genes between both T cell subpopulations (delta = 2.15, median FDR = 0.18). After entering the generated data set into Genesis software, a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory T cells in RCC patients from those of healthy donors. It consists of 71 up-regulated and 64 down-regulated genes.

3.4.2.3 Metastatic *versus* localized RCC Microarray analyses of T_{Reg} and naive T cells obtained from seven and five RCC patients with localized and metastatic RCC growth, respectively, allowed direct comparison of resulting gene expression profiles depending on stage of disease. As depicted in Figure 34, 72 and 85 genes were revealed to be significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells in localized and metastatic RCC, respectively. Among them, FOXP3, IL2RA and CTLA4 could be again confirmed as "old friends" in T_{Reg} cell immunology, but various "new players" - such as BCL2, CCR6 and ITGB5 - were additionally detected that have not been described in T_{Reg} cell context before.

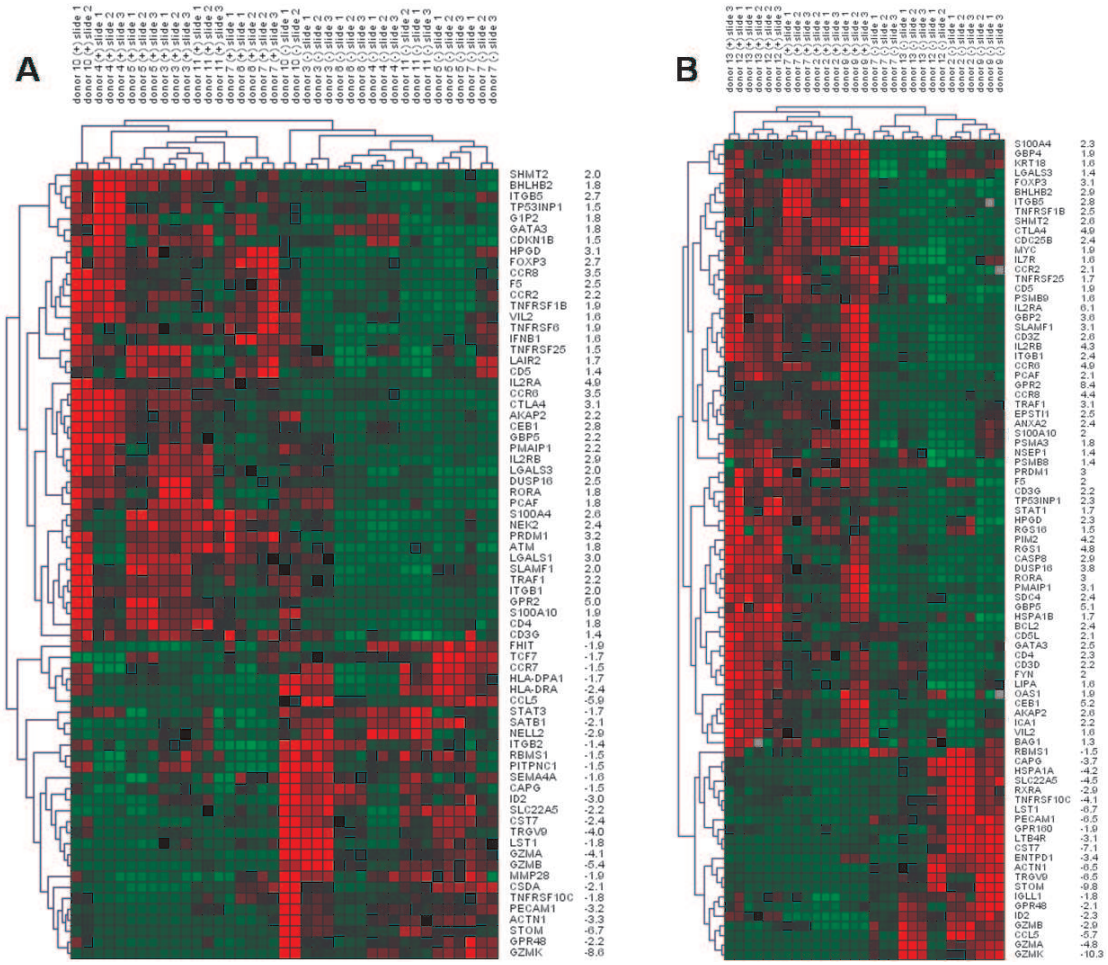

Figure 34: Gene expression profiling of $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells from RCC patients with localized (A) and metastatic (B) tumor growth.
To reveal molecular differences between regulatory and naive human T cells in RCC patients with localized (A) and metastatic (B) tumor growth, differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, SAM was applied as a data mining tool to ascertain gene expression changes, disclosing 72 (A) and 85 (B) significantly altered genes between both T cell subpopulations (A: delta = 1.38, median FDR = 0.8; B: delta = 2.1, median FDR = 0.5). After entering the generated data set into Genesis software a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory from naive T cells in localized (A) or metastatic (B) RCC consisting of 43 (A) or 63 (B) up-regulated and 29 (A) or 22 (B) down-regulated genes.

The Venn diagram in Figure 35 summarizes the results of a comparison of both transcriptional patterns. As depicted by the intersection, 51 genes were uncovered to be differentially expressed between T_{Reg} and T_{naiv} cells in localized as well as in metastatic RCC. Similarly to observations made between healthy donors and RCC patients, most of these genes were stronger regulated in metastic than localized tumor growth and opposite directions of regulation were not discovered. Additional 21 and 34 genes, respectively, were found to be an exclusive part of the expression profile from either localized or metastatic RCC.

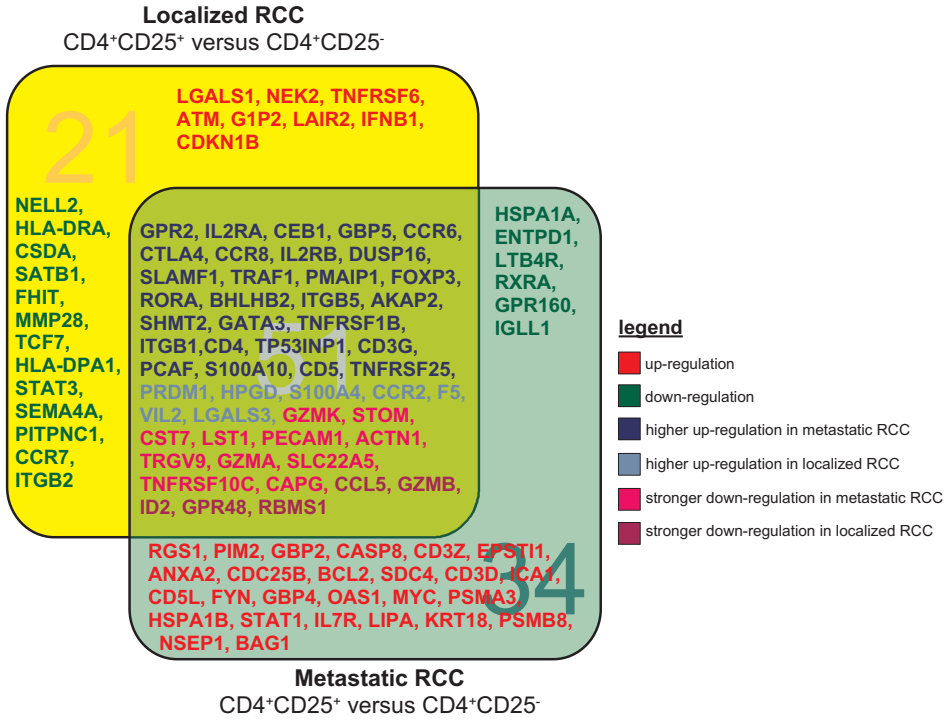

Figure 35: Similarities and differences in the gene expression signatures of T_{Reg} cells in localized and metastatic RCC.
Genes significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells from seven and five RCC patients suffering from localized and metastatic tumor growth, respectively, were summarized in a Venn diagram. As depicted by the intersection, 51 genes were contained in the expression profiles of both donor groups, but generally showed stronger regulation in metastatic RCC. Additional 21 and 34 genes, respectively, were found to be just differentially expressed between T_{Reg} and naive T cells of either localized or metastatic RCC. Genes were arranged according to their decreasing fold change between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells.

3.4.3 More news about the phenotype of human T_{Reg} cells in RCC

3.4.3.1 Confirmation of microarray data Quantitative real-time RT-PCR was performed using the original RCC samples to verify the obtained microarray data. Referring to FOXP3 as a well-characterized T_{Reg} cell gene, the applied approach could be confirmed [Figure 36A]. This gave greater credence and reliability to the numerous

additional genes that have not yet been reported in (human) T_{Reg} cells. Four of the "new players" were selected that have also been identified as differentially expressed in $CD4^+CD25^+$ T_{Reg} *versus* $CD4^+CD25^-$ T cells of healthy donors (LGALS3, CCR10, TNFRSF1B, TRAF1) and their T_{Reg} cell specific expression was approved by quantitative real-time RT-PCR [Figure 36B-E]. As depicted and shown for the healthy donors before, PCR results generally correlated well with the differential gene expression data obtained by application of the *Human T_{Reg} Chip*. For a few donors variability in gene expression was observed between microarray and quantitative RT-PCR data, but the direction of change was consistent, lending confidence to the reliability of the *Human T_{Reg} Chip* results and the applied cell separation strategy. While similar fold changes in mRNA expression were observed for FOXP3, CCR10, TNFRSF1B and TRAF1 in T_{Reg} *versus* naive T cells comparing healthy to RCC donors, a significant decrease was detected in the fold change of LGALS3 expression in RCC patients [Figure 36F]. In healthy donors, LGALS3 was 7.6 ± 3.4 times over-expressed in T_{Reg} *versus* naive T cells, whereas this fold change declined to 2.9 ± 1.0 in RCC patients when comparing the same T cell subsets. These results directly reflected the microarray data which revealed fold changes of 3.9 ± 1.5 for healthy donors and 2.9 ± 1.0 for RCC patients. For all the genes analysed by real-time RT-PCR, no significant differences in expression levels could be observed between localized and metastatic RCC tumor growth.

3.4.3.2 Protein expression of human T_{Reg} cells in RCC Next, correlation of FOXP3, LGALS3 and TNFRSF1B mRNA and protein expression was examined because post-transcriptional gene regulation mechanisms may influence mRNA stability. Therefore, three-colour staining with subsequent FACS analysis was applied to PBMCs isolated from seven RCC patients that were additionally gated for $CD4^+CD25^{high}$ T_{Reg} and $CD4^+CD25^-$ naive T cells. Obtained results were directly compared to accordant findings derived from previous analyses of nine healthy donors [chapter 3.3.4.2 and 3.3.4.3]. As illustrated in Figure 37A and comparable to healthy donors, FOXP3 and LGALS3 protein expression levels were significantly elevated in RCC T_{Reg} *versus* naive T cells. Furthermore, very similar levels of FOXP3 protein expression were observed in $CD4^+CD25^{high}$ T_{Reg} cells of healthy (84.3% ±3.5%) and RCC (84.9% ± 5.7%) donors [Figure 37B]. In contrast to that, significant elevated fractions of LGALS3 expressing cells were detected within the T_{Reg} subpopulation in RCC patients compared to healthy volunteers. In average, 23.5% ± 7.0% of $CD4^+CD25^{high}$ T_{Reg} cells isolated from healthy donors were LGALS3 positive, whereas this percentage rose to 37.6% ± 8.3% in RCC patients ($P < 0.004$). This is well in line with the obtained *Human T_{Reg} Chip* expression profile comparing healthy and RCC T_{Reg} cells which also reported a 2.2 times higher LGALS3 expression in T_{Reg} cells isolated from RCC patients [Figure 33]. Next, the extent of coexpression of FOXP3 and either LGALS3 or TNFRSF1B was investigated. Comparing healthy donors to RCC patients again, significant larger $CD4^+$ T cell subpopulations were revealed coexpressing LGALS3 and FOXP3 as well as TNFRSF1B and FOXP3 in the RCC donor group [Figure 37]. While only 1.5% ± 0.4% of healthy

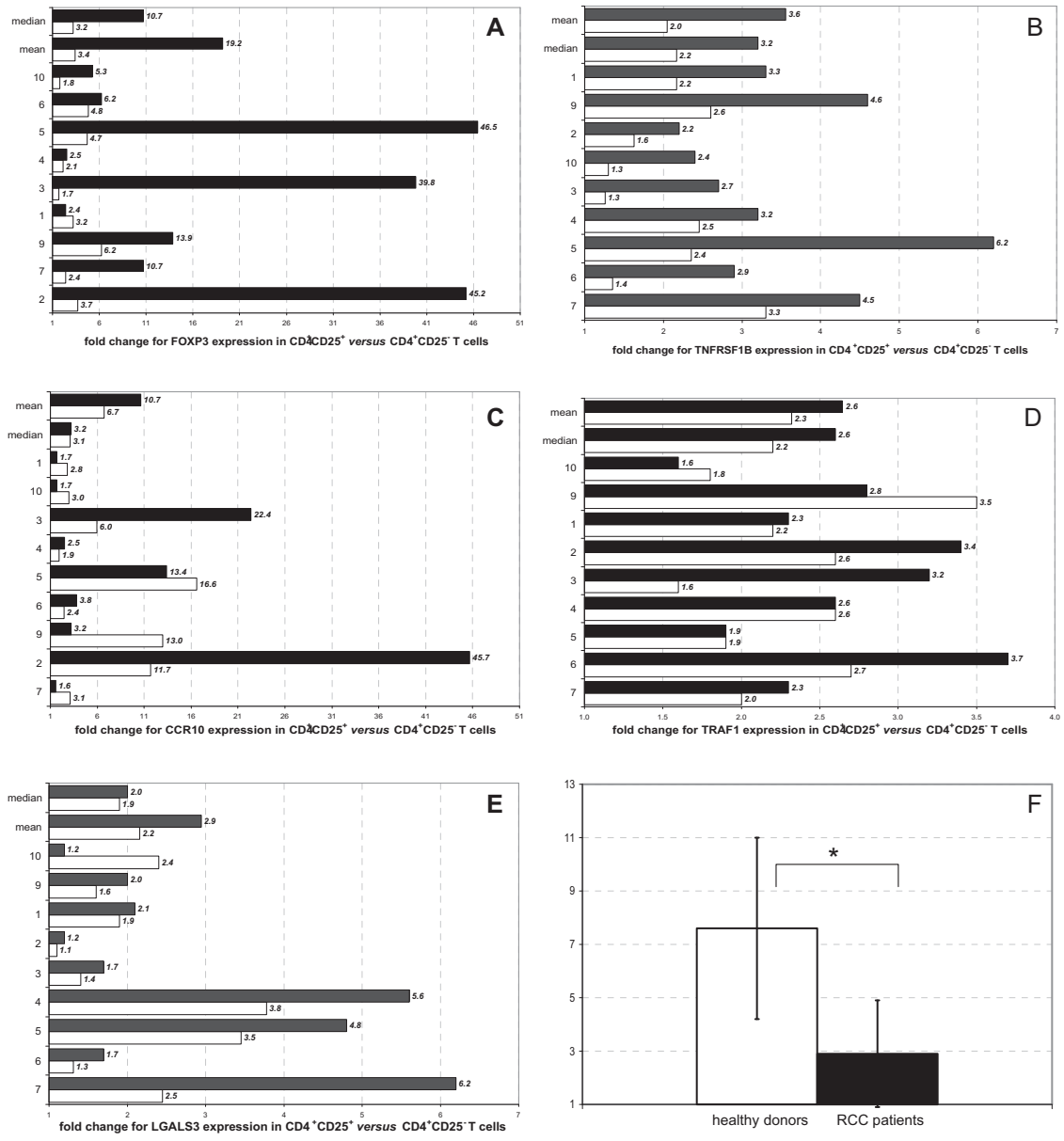

Figure 36: Confirmation of microarray results in RCC.
Real-time RT-PCR was performed for FOXP3 (A), TNFRSF1B (B), CCR10 (C), TRAF1 (D), LGALS3 (E), and RPS9 (data not shown) expression in MACS separated human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells isolated from RCC patients. Following normalization to RPS9, relative mRNA amounts in $CD4^+CD25^+$ T_{Reg} cells were adjusted to corresponding expression levels in $CD4^+CD25^-$ naive T cells and expressed as fold changes. Real-time RT-PCR results, indicated by black bars, were compared to fold changes arising from the *Human T_{Reg} Chip* (represented by white bars). The particular RCC patients are specified by letters according to Table 1. Furthermore, mean and median fold changes for all indicated volunteers are given for each gene. (F) illustrates the significant decrease in the fold change of LGALS3 expression in $CD4^+CD25^+$ T_{Reg} *versus* $CD4^+CD25^-$ naive T cells from RCC patients compared to healthy donors (*P = 0.006).

$CD4^+$ T cells coexpressed LGALS3 and FOXP3, this fraction increased to 2.5% ± 0.9% in RCC patients (P = 0.03). Similarly, 0.5% ± 0.2% of $CD4^+$ T cells isolated from healthy donors showed coexpression of TNFRSF1B and FOXP3, whereas this population comprises 1.2% ± 0.7% $CD4^+$ T cells in RCC patients (P = 0.04). The latter increase was accompanied by a significant rise in the fraction of $CD4^+$ $TNFRSF1B^+$ T cells lacking FOXP3 expression in RCC patients (10.9% ± 3.7%) compared to healthy controls (5.8% ± 2.3%; P = 0.01).

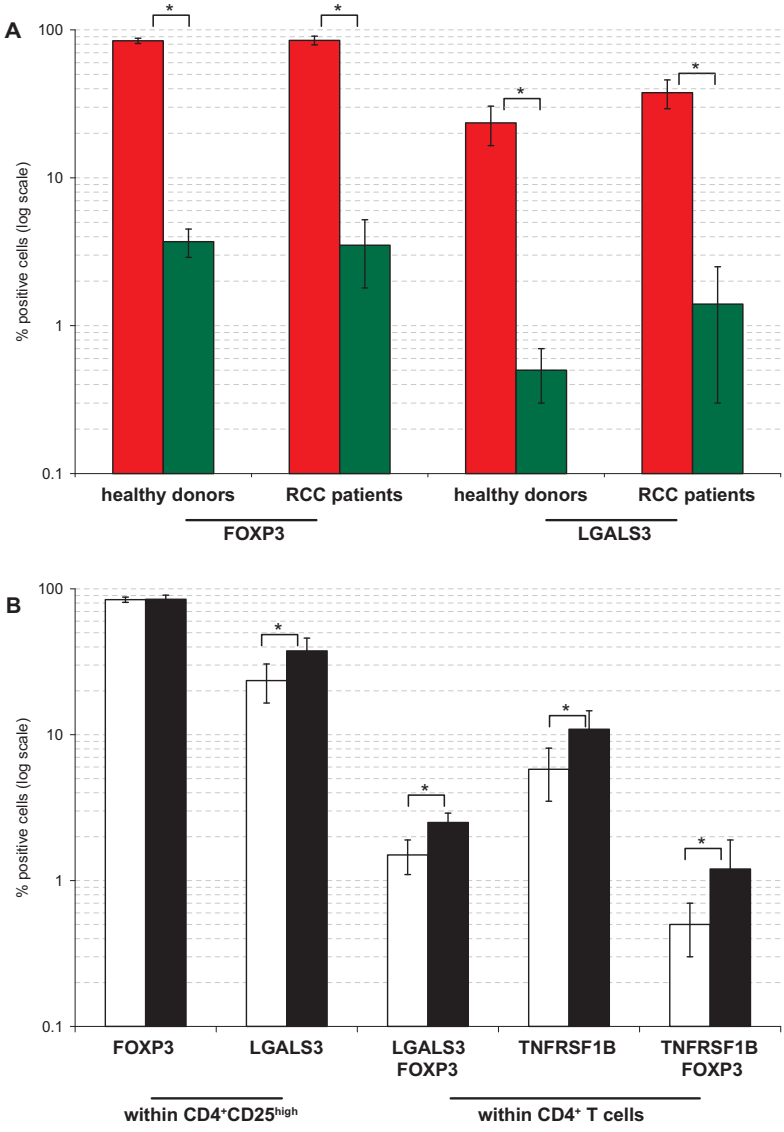

Figure 37: Protein expression of human T_{Reg} cells in health and RCC. PBMCs of nine healthy and seven RCC donors were isolated, stained for CD4, CD25, FOXP3, TNFRSF1B and LGALS3. **A** Elevated expression of FOXP3 and LGALS3 proteins in RCC T_{Reg} (red bars) *versus* naive (green bars) T cells, comparable to that observed in healthy donors. (*P < $2.1 \cdot 10^{-5}$). **B** Gated $CD4^+CD25^{high}$ T_{Reg} were analysed for either FOXP3 or LGALS3 protein expression by FACS. Alternatively, PBMCs were gated for total $CD4^+$ T cells and fraction of cells coexpressing TNFRSF1B and FOXP3 as well as FOXP3 and LGALS3 were determined. Percentages of cells expressing the proteins-of-interest in the different T cell subpopulations were compared between healthy donors (white bars) and RCC patients (black bars; *P < 0.04). Values are represented in logarithmic scale as means ± standard deviation.

3.4.3.3 Susceptibility to apoptosis As discussed in more detail in chapter 4.3.2, the performed microarray analyses revealed several genes participating in apoptosis control. Using real-time RT-PCR [Figure 36] as well as flow cytometry [Figure 37], up-regulation of two anti-apoptotic genes/proteins, namely LGALS3 and TNFRSF1B, could be confirmed on human $CD4^+CD25^{high}$ T_{Reg} compared to naive $CD4^+CD25^-$ T cells isolated from RCC patients. To elucidate whether both T cell subpopulations consequently differ in their apoptotic susceptibility, a combined Annexin V and propidium iodide staining of isolated PBMCs with subsequent FACS analysis was carried out and results obtained were directly compared to those received from healthy donors. Gating for $CD4^+CD25^{high}$ T_{Reg} and naive $CD4^+CD25^-$ T cells indeed revealed that regulatory T cells are less susceptible to apoptosis than their naive counterparts, in fact independent from their donor origin [Figure 38]. As illustrated, a fraction of 2.6% ± 1.1% T_{Reg} *versus* 5.9% ± 1.0% naive T cells for healthy donors and 2.9 ± 1.4% T_{Reg} *versus* 9.2% ± 3.4% naive T cells from RCC patients were demonstrated to undergo apoptosis. These differences in apoptotic behaviour were found to be highly significant (healthy: $P < 10^{-4}$; RCC: $P < 0.005$). Nevertheless, the number of apoptotic cells did not vary significantly when comparing T_{Reg} cells isolated either from healthy or RCC donors. Instead, naive T cells showed clearly higher apoptotic rates in RCC patients than in healthy donors. This result bordered on significance ($P = 0.06$) since it was found to be variable among RCC donors, but should be further analysed by increasing the numbers of RCC patients in this study.

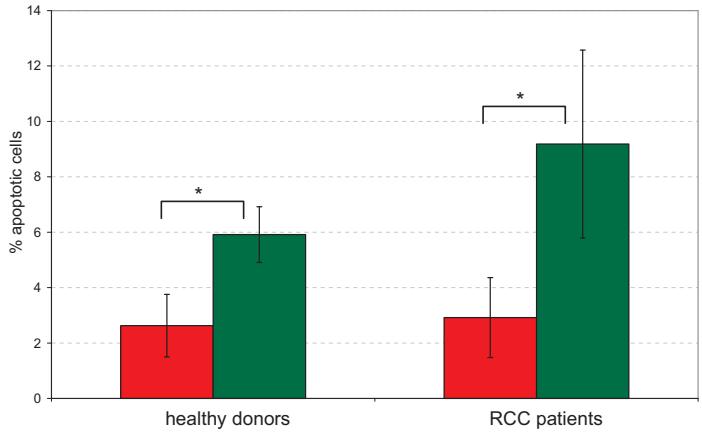

Figure 38: Similar to human $CD4^+CD25^{high}$ T_{Reg} cells from healthy donors, RCC patient derived T_{Reg} cells are less susceptible to apoptosis than their naive counterparts.
PBMCs from seven healthy and six RCC donors were isolated and stained for CD4, CD25 as well as with Annexin V and propidium iodide (PI). These cells were analysed by FACS, gated for lymphocyte population first and afterwards for $CD4^+CD25^{high}$ T_{Reg} (red bars) and $CD4^+CD25^-$ naive (green bars) T cells, respectively. Percentages of apoptotic cells, which are Annexin V^+ but PI^- are given as means ± standard deviation (*$P < 0.005$).

3.5 Human regulatory T cells in IBD

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory condition of the gastrointestinal tract that manifests at two distinct but sometimes overlapping clinical entities, known as Crohn's disease (CD) and ulcerative colitis (UC). T_{Reg} cells have been proven effective in the prevention and down-regulation of IBD in animal models, thus abnormalities in their suppressor function, frequency or distribution in the intestinal mucosa are supposed to contribute to IBD pathogenesis [235]. These facts led us to have a closer look on T_{Reg} cells isolated from IBD patients, suffering either from UC or CD and presenting active or inactive state of disease.

3.5.1 Frequency of T_{Reg} cells in IBD patients

In a cooperation project with Dr. Jochen Maul under the direction of Prof. Duchmann at the Charité-University Medicine Berlin, T_{Reg} cells isolated from PB of 36 patients with active and 34 patients with inactive CD or UC have been investigated. As published during this work, changes in T_{Reg} cell frequency were revealed in IBD that additionally correlated with disease activity [235]. Maul *et al.* showed decreased PB T_{Reg} cell numbers in active IBD, while T_{Reg} cells were enriched in the PB of IBD patients in remission (inactive state). Although, T_{Reg} cell frequency was enhanced in inflamed active IBD lesions *versus* noninflamed areas within the intestinal mucosa, their number remained significantly lower compared to diverticulitis, an inflammatory control expected to exhibit undisturbed immunoregulatory functions. Thus, active IBD seems to be characterized by an insufficient counterregulation, i.e. contraction of the peripheral $CD4^+CD25^+$ T_{Reg} cell pool and an insufficient increase of their frequency in intestinal lesions [235].

3.5.2 Gene expression profile by application of the *Human T_{Reg} Chip*

To decipher gene regulation events and to identify novel genes that might be involved in IBD pathogenesis, maintenance and progression, comparative microarray analyses were performed. As described previously, $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells were therefore isolated from PB of IBD patients suffering from active CD, inactive CD or inactive UC (n = 3 each). IBD patients' characteristics were summarized in Table 1. Total RNA was extracted and reverse transcribed into cDNA. In subsequent IVT reactions using T7 RNA polymerase, cRNA could be amplified and hybridized to the *Human T_{Reg} Chip*. Altogether, 18 microarray experiments were carried out; 9 slides were hybridized with $CD4^+CD25^+$ and another 9 with $CD4^+CD25^-$ T cell-derived cRNA, respectively.

3.5.2.1 Regulatory *versus* naive T cells in IBD To identify genes significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells in IBD, microarray data was normalized and subjected to SAM and cluster analysis as previously

described. The revealed transcriptional pattern arranged coexpressed genes and replicated experiments next to each other and clearly separated $CD4^+CD25^+$ T_{Reg} from $CD4^+CD25^-$ T cells. The gene expression signature of regulatory and naive T cells in IBD turned out to consist of 64 altered genes, which were composed of 40 up-regulated and 24 down-regulated candidates [Figure 39]. Among them, prominent ones were expectedly found, such as FOXP3, CTLA4 and IL2RA, but also numerous genes not previously associated with T_{Reg} cells at all (e.g. PECAM1, CCR8, CCL5).

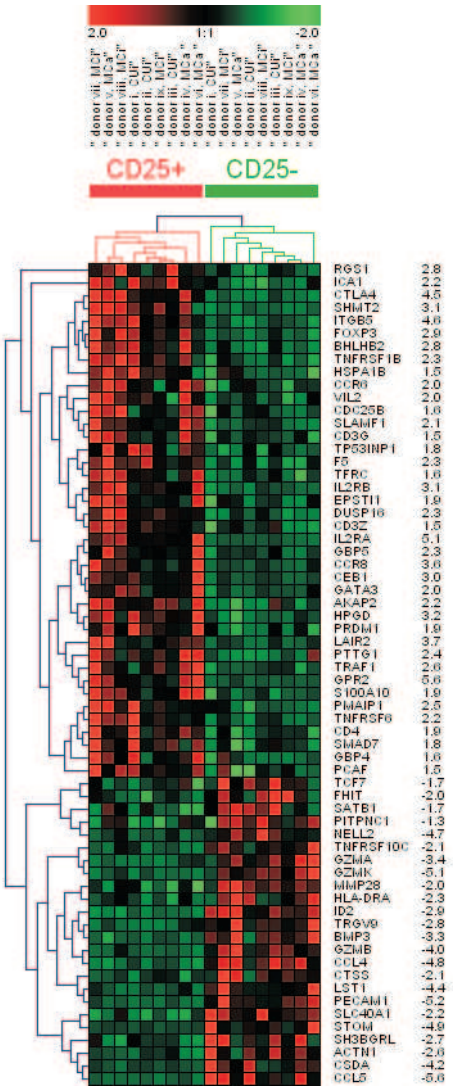

Figure 39: Transcriptional profiling of $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells from IBD patients.
To reveal molecular differences between regulatory and naive human T cells in IBD patients differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, SAM was applied as a data mining tool to ascertain gene expression changes, disclosing 64 significantly altered genes between both T cell subpopulations (delta = 1.57, median FDR = 0.99). After entering the generated data set into Genesis software, a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory from naive T cells in IBD consisting of 40 up-regulated and 24 down-regulated genes. (MCa = Crohn's disease active state, MCi = Crohn's disease inactive state, UCi = ulcerative colitis inactive state)

3.5.2.2 Comparison to healthy donors and RCC patients As changes in frequency and insufficient regulatory activity of T_{Reg} cells in IBD would be reflected in the revealed gene expression profile, it was compared to the transcriptional pattern obtained from T_{Reg} and naive T cells of healthy donors [Figure 20] functioning as a reference fingerprint. Although no genes with opposed regulations could be revealed between both donor groups, numerous genes were detected that were just differentially expressed between T_{Reg} and naive T cells of either healthy donors (n = 30) or IBD patients (n = 32, [Figure 40]).

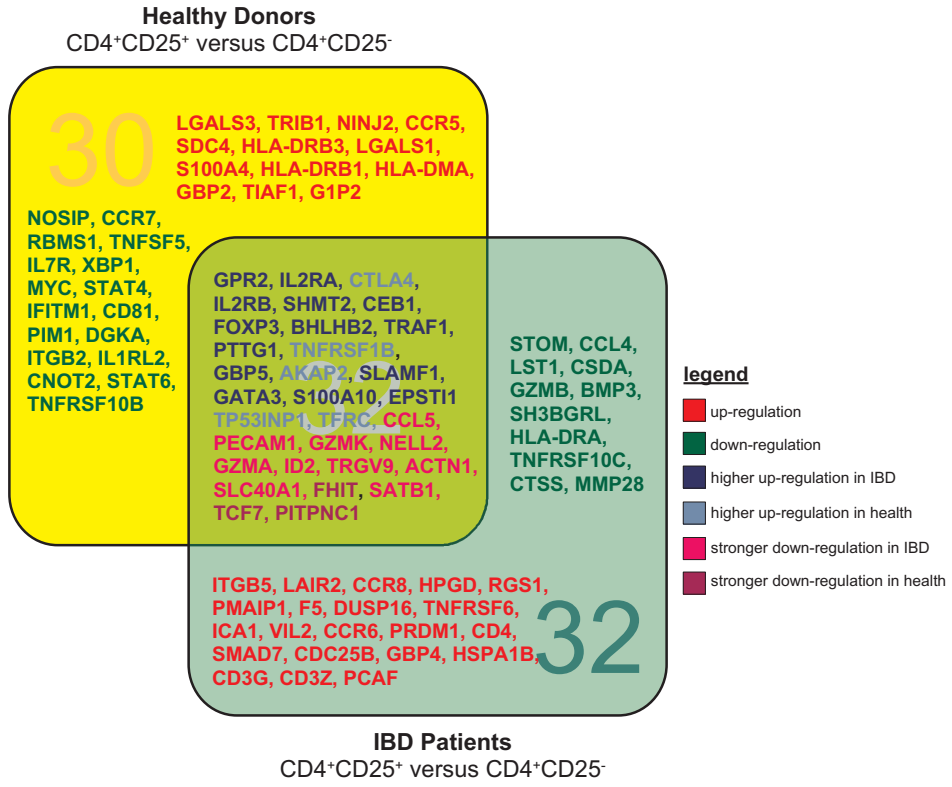

Figure 40: Similarities and differences in the gene expression signatures of T_{Reg} cells in IBD and health.
Genes significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells from 9 IBD patients and 11 healthy volunteers, respectively, were summarized in a Venn diagram. As depicted by the intersection, 32 genes were contained in the expression profiles of both donor groups, but generally showed stronger regulation in IBD. Additional 30 and 32 genes, respectively, were found to be just differentially expressed between T_{Reg} and naive T cells of either healthy donors or IBD patients. Genes were arranged according to their decreasing fold change between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells.

Additional 32 genes were included in the expression profiles from T_{Reg} and naive T cells of both donor groups, but generally showed stronger regulation in IBD. Interestingly, a comparison of this Venn diagram to the one obtained by analysis of RCC patients [compare Figure 32] revealed that the composition of genes exclusively altered between T_{Reg} and naive T cells in healthy donors remained almost unchanged, thus indicating their importance for the maintenance of a healthy T_{Reg} cell phenotype. In

contrast to that, genes uniquely changed between T_{Reg} and naive T cells in disease differed considerably between RCC and IBD patients, underlining distinct differences in T_{Reg} cell gene expression for both disorders.

To reveal significant differences in the gene expression profiles of $CD4^+CD25^+$ T_{Reg} between healthy volunteers and IBD patients, microarray data of the same cell type but of different donor origin had to be compared. As illustrated in Figure 41, SAM and hierarchical clustering analysis identified 109 genes differentially expressed in T_{Reg} cells of both donor groups, composed of 35 up-regulated and 74 down-regulated genes when comparing IBD to health. In contrast to previously shown expression profiles, the separation of healthy from IBD T_{Reg} cells was not comparably clear as illustrated by the chosen clustering behaviour. This result is probably caused by the patients presenting with inactive state of IBD. As they are in remission, this could reflect an increasing rapprochement of T_{Reg} cell gene expression to healthy standard thus hampering proper classification. Comparison of this IBD T_{Reg} cell fingerprint to the one obtained by analysis of RCC patients [see Figure 31] revealed a significantly lower number of up-regulated genes in IBD *versus* RCC patients that additionally showed weaker over-expression. In exchange, more genes were detected to be down-regulated in a stronger manner in IBD patients, although the total number of altered genes was higher in RCC compared to IBD. These findings support ideas of a gain-of-function in T_{Reg} cells from RCC patients that may mediate enhanced suppressive capacities enabling tumor immune escape, whereas increased down-regulation of T_{Reg} cell genes could point to loss-of-functions contributing to insufficient regulatory features and thus to IBD pathogenesis.

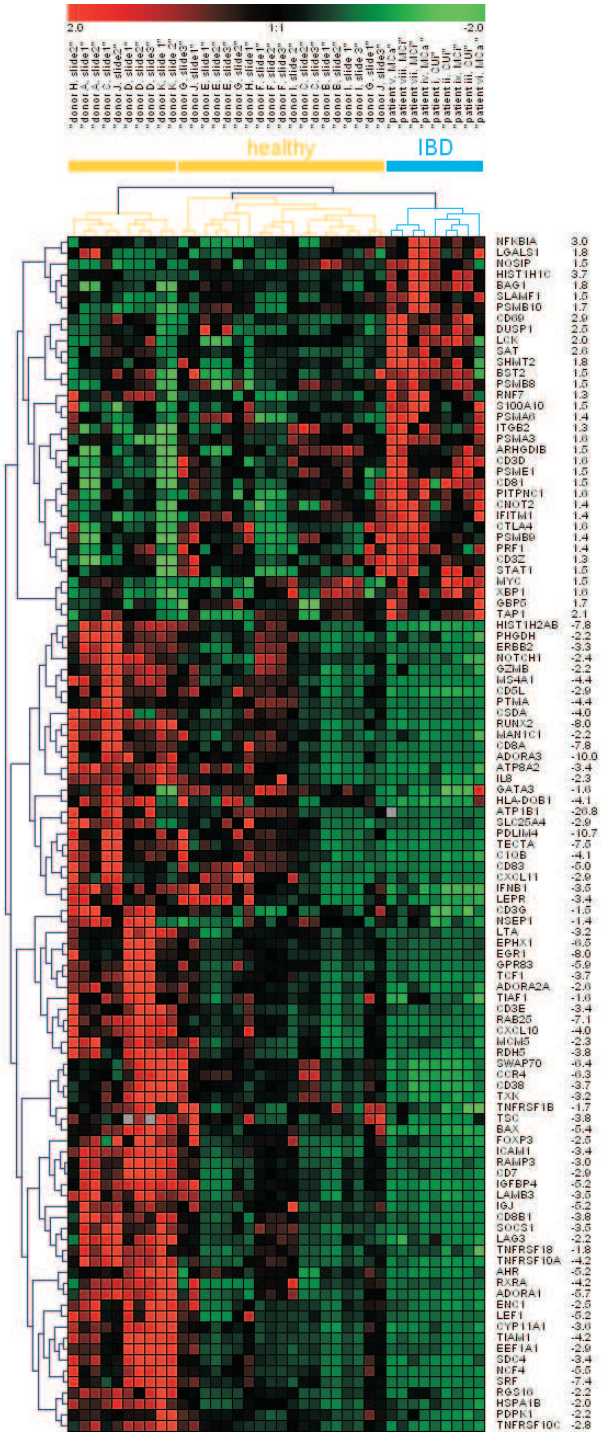

Figure 41: Expression profiling of $CD4^+CD25^+$ T_{Reg} from IBD patients and healthy donors.

To reveal molecular differences between regulatory T cells in IBD patients compared to healthy donors differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, SAM was applied as a data mining tool to ascertain gene expression changes, disclosing 68 significantly altered genes between both T cell subpopulations (delta = 1.41, median FDR = 0.2). After entering the generated data set into Genesis software a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory T cells in IBD patients from those of healthy donors. It consists of 35 up-regulated and 74 down-regulated genes.

3.5.2.3 Active *versus* inactive state of disease and CD *versus* UC Next, separate comparison of T_{Reg} *versus* naive T cells from (in)active CD and inactive UC patients enabled definition of differences and similarities in T_{Reg} cell gene expression depending on IBD stage. As illustrated in Figure 42, the number of differentially expressed genes between both T cell subpopulations differed considerably between patient cohorts dependent on disease stage: Whereas 58 altered genes (41↑, 17↓) were revealed in patients suffering from active CD, this number decreased *via* 42 (28↑, 14↓) for inactive UC to only 34 (17↑, 17↓) in inactive CD.

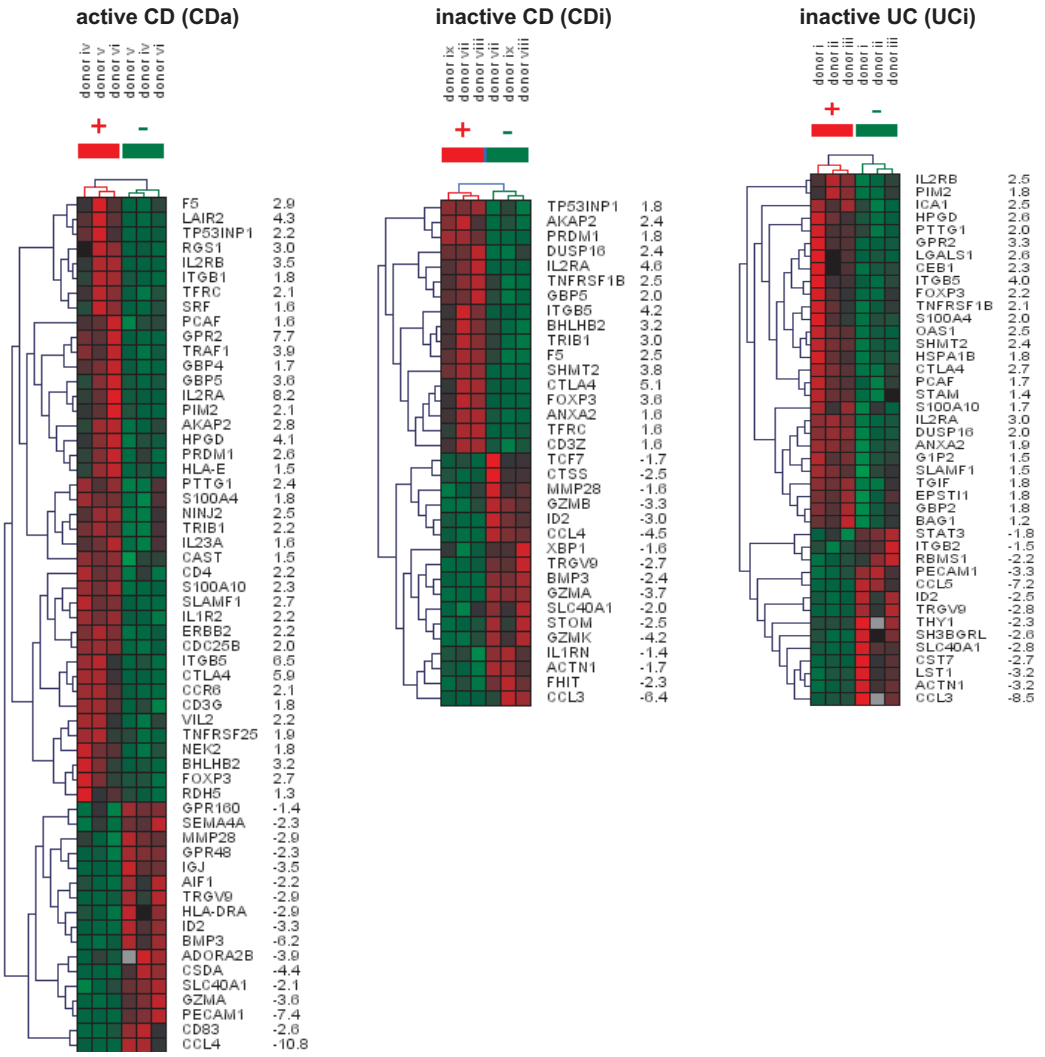

Figure 42: Gene expression profiling of $CD4^+$ $CD25^+$ T_{Reg} *versus* $CD4^+$ $CD25^-$ naive T cells from IBD patients with active CD (left), inactive CD (middle) and inactive UC (right).
To reveal molecular differences between regulatory and naive human T cells in distinct IBD states differential expression of 350 genes was investigated by application of the *Human T_{Reg} Chip*. Following data normalization, SAM was applied as a data mining tool to ascertain gene expression changes, disclosing 58 (CDa), 37 (CDi) and 42 (UCi) significantly altered genes between both T cell subpopulations (CDa: delta = 1.08, median FDR = 1.53; CDi: delta = 1.35, median FDR = 1.09; UCi: delta = 0.65, median FDR 1.52). After entering the generated data set into Genesis software, a two-dimensional hierarchical clustering analysis uncovered the displayed transcriptional pattern discriminating human regulatory from naive T cells in the investigated IBD stages. Grey cells indicate missing signal intensity values.

The Venn diagram in Figure 43 summarizes the results of a comparison of the three transcriptional patterns. As depicted by the intersection, only 7 genes were uncovered to be differentially expressed between T_{Reg} and T_{naiv} cells in CD *versus* UC and inactive *versus* active state of disease. Similarly to observations made between healthy donors and IBD patients, six of these genes showed strongest regulation in active CD, with decreasing fold changes *via* inactive CD to inactive UC, supporting the idea of a rapprochment of T_{Reg} cell gene expression to healthy values. Opposite directions of regulation were not discovered. Furthermore, the depicted Venn diagram allows identification of T_{Reg} cell genes that either distinguish or unite (inactive) CD from (inactive) UC, active from inactive CD and active CD from inactive UC. Expectedly, active IBD is characterized by the highest number of genes differentially expressed between T_{Reg} and naive T cells in the difference section (29), whereas the number of regulated genes in the particular section decreased *via* inactive UC (19) to only 9 in inactive CD.

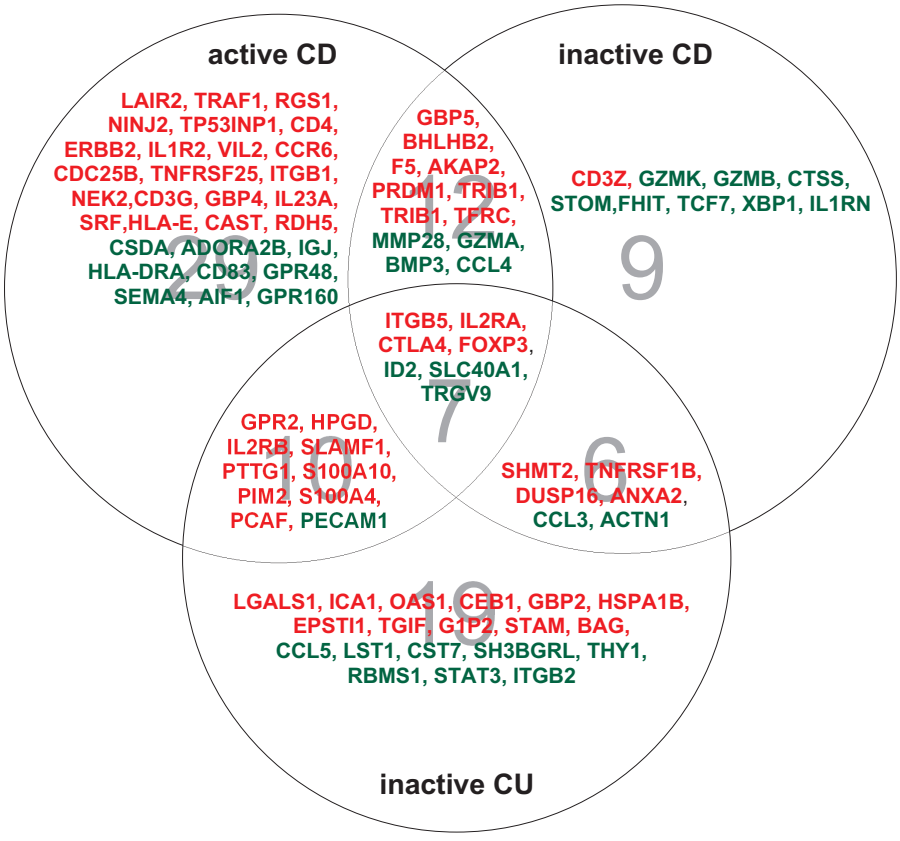

Figure 43: Similarities and differences in the gene expression signatures of T_{Reg} cells in active CD, inactive CD and inactive UC.
Genes significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells from IBD patients suffering active CD, inactive CD and inactive UC, respectively, were summarized in a Venn diagram. As depicted by the intersection, 7 genes were contained in the expression profiles of all three patient cohorts, but generally showed strongest regulation in active CD. Genes were arranged according to their decreasing fold change between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells; red indicates up-regulated, green means down-regulated genes.

3.5.3 Confirmation of microarray data by quantitative real-time RT-PCR

As done before for healthy donors and RCC patients, quantitative real-time RT-PCR was performed using the original IBD samples to verify the obtained microarray data. Referring to FOXP3 as a well-characterized T_{Reg} cell gene, the approach could be additionally confirmed for LGALS3, CCR10, TNFRSF1B and TRAF1 gene expression [Figure 44A-E]. Although LGALS3 was excluded by SAM algorithm to be significantly differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ T cells in IBD patients, comparison of fold changes between healthy donors and IBD patients as obtained by real-time RT-PCR revealed a significant down-regulation in IBD (healthy: 7.6 ± 3.4 *versus* IBD: 1.4 ± 0.8, $P = 9.9 \cdot 10^{-4}$, Figure 44F). Similarly, a significant decrease in the fold change of TNFRSF1B gene expression was observed in IBD patients (healthy: 4.8 ± 1.7 *versus* IBD: 2.9 ± 1.8, $P = 0.04$), whereas FOXP3 mRNA levels expressed as fold changes showed a significant increase in IBD patients (healthy: 11.5 ± 8.7 *versus* IBD: 29.9 ± 17.2, $P = 0.02$). Comparing these real-time RT-PCR results between RCC and IBD patients as well as between active and inactive state of IBD did not reveal significant differences between patient groups.

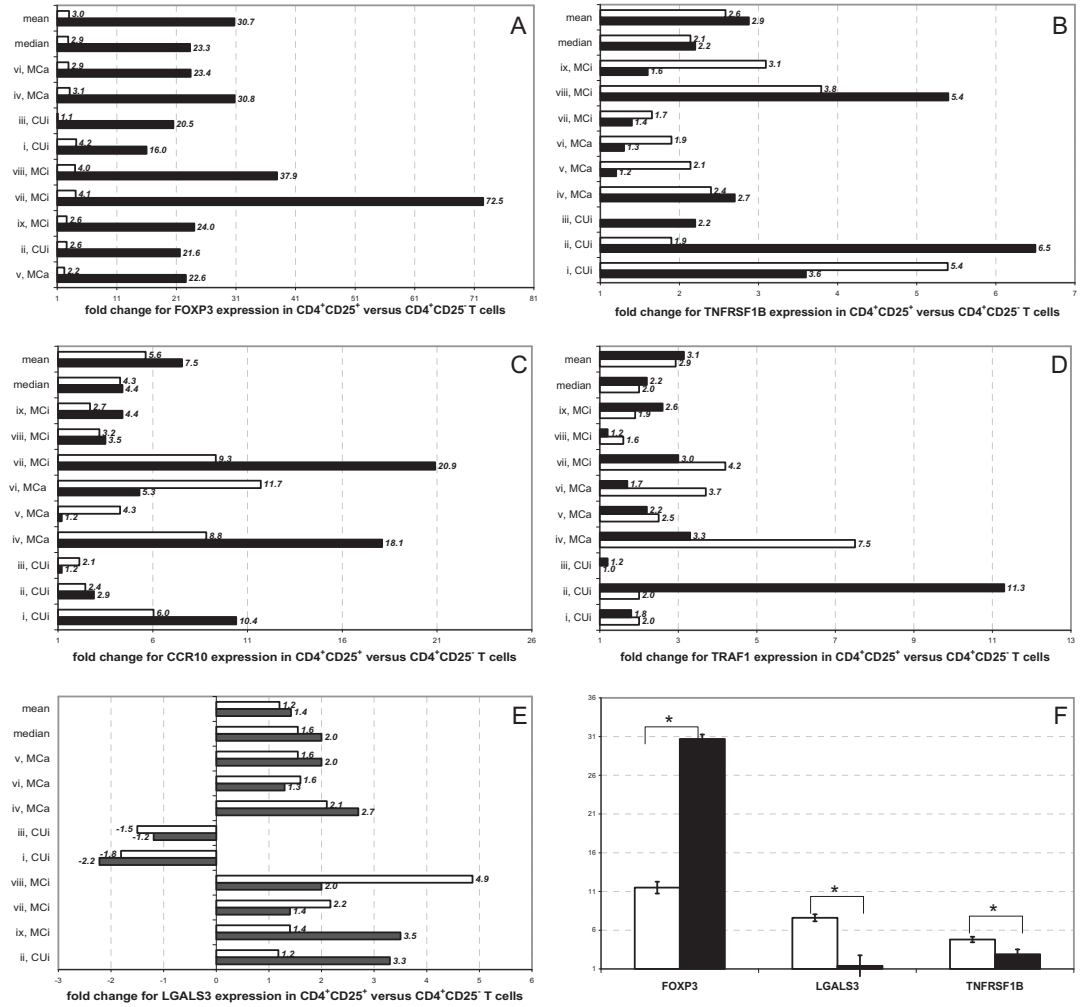

Figure 44: Confirmation of microarray results in IBD.
Real-time RT-PCR was performed for FOXP3 (A), TNFRSF1B (B), CCR10 (C), TRAF1 (D), LGALS3 (E), and RPS9 (data not shown) expression in MACS separated human $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells isolated from IBD patients. Following normalization to RPS9, relative mRNA amounts in $CD4^+CD25^+$ T_{Reg} cells were adjusted to corresponding expression levels in $CD4^+CD25^-$ naive T cells and expressed as fold changes. Real-time RT-PCR results, indicated by black bars, were compared to fold changes arising from the *Human T_{Reg} Chip* (represented by white bars). The particular IBD patients are specified by letters according to Table 1. Furthermore, mean and median fold changes for all indicated volunteers are given for each gene. (F) illustrates the significant changes in the fold change of LGALS3, FOXP3 and TNFRSF1B expression in $CD4^+CD25^+$ T_{Reg} *versus* $CD4^+CD25^-$ naive T cells from IBD patients (■) compared to healthy donors (□, *$P < 0.04$).

4 Discussion

4.1 Performance of the *Human T_{Reg} Chip*

To ensure measurement of accurate and reliable transcription profiles, the *Human T_{Reg} Chip* has been validated in terms of cross-platform comparison, sensitivity and reproducibility. First, a concordance of 81% could be demonstrated for the detection of significantly regulated genes generated with the *Human T_{Reg} Chip* and Affymetrix GeneChips, respectively. The question of cross-platform agreement for gene expression studies has been previously addressed by several investigators, but without clear consensus [107]. Some groups claimed a significant divergence comparing spotted oligonucleotide arrays (30- to 80-mers) and Affymetrix GeneChips® by reporting correlation coefficients of < 0.6 [108, 109] or even only 0.21 [106], while others described acceptable concordance levels of 80-90% for such a platform comparison [110, 111]. Furthermore, it was observed that sequence-matched probes increase cross-platform consistency resulting in a correlation coefficient of ~ 0.7 for a comparison between Affymetrix and Agilent 60mer oligonucleotide microarrays [112]. Thereby, application of different target preparation methods and array surface chemistries, may in part be responsible for discrepancies in gene expression measurements across microarray platforms. Taken together, the developed *Human T_{Reg} Chip* demonstrated high comparability to the Affymetrix approach, showing a correlation coefficient at the upper range of those described in literature. Next, reproducibility of measurements as another quality parameter of the *Human T_{Reg} Chip* was assessed. Therefore, identical samples were applied to different *Human T_{Reg} Chip*s and signal intensities were compared to each other. The median correlation coefficient yielded from 52 log-log-plots was 0.98, emphasizing a high degree of internal consistency and reliability of the applied microarray system [113]. Customized oligonucleotide microarrays have been reported to show average correlation coefficients for repeated experiments of ~ 0.9 [108], while significantly lower reliability was obtained for pin-spotted arrays [114]. Affymetrix' reproducibility has been described as highly reliable, expressed in pairwise correlation coefficients ranging from 0.96 to almost 1 [115, 116]. To summarize, the *Human T_{Reg} Chip* provides reliability similar to Affymetrix and previously published oligonucleotide microarrays. Furthermore, the accuracy of measurements was determined expressed as coefficient of variance calculated across eight replicates per gene. It could be demonstrated, that the vast majority of signal intensities (74%) calculated for the entire data set varied less than 30% reflecting the robustness of the applied microarray approach. Finally, bacterial control genes at different concentrations were used to monitor microarray system sensitivity and the spectrum of linear signal measurement. A final concentration of 0.3 pM was reliably detectable corresponding to one transcript in 500,000 or approximately one copy per cell. Furthermore, a linear regression could be demonstrated between signal intensity and concentration covering more than three orders of magnitude. By considering all these results of the evaluation process, the *Human T_{Reg} Chip* seems to

represent a reliable and suitable tool facilitating research of human regulatory T cells. Furthermore, it merges several advantages. Due to its inexpensive production costs it enables extensive microarray analyses, like e.g. investigation of samples from individual donors, thereby avoiding sometimes misleading probe pools and delivering data reflecting similarities as well as individual differences. Additionally, experiments can be repeated in more replicates, allowing calculation of statistical parameters leading to increased reliability of the obtained data. Moreover, customized microarrays, such as the *Human T_{Reg} Chip*, provide a more focussed view at the particular question under examination, thereby preventing unmanageably huge floods of data. To summarize, the *Human T_{Reg} Chip* enables broad microarrays analyses at a higher resolution and lower costs.

4.2 Regulatory T cells in health

4.2.1 Purity of isolated cell fractions and frequency of T_{Reg} cells

Reliable and significant transcription profiles of human $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells can only be obtained if isolation of highly pure T cell subpopulations can be assured. Both T cell fractions were separated from peripheral blood of healthy donors by MACS at purities consistently greater than 90% for T_{Reg} and more than 85% for naive T cells, respectively. Although, constitutive expression of CD25 is a primary characteristic of human T_{Reg} cells that allows consistent isolation of functionally suppressive cells from human peripheral blood, CD25 alone is not a reliable marker for T_{Reg} cells in humans [55]. While mouse T_{Reg} cells appear as a distinct $CD4^+CD25^+$ T cell population that can easily be distinguished from their naive counterparts, human $CD4^+$ T cells exhibit a more continuous and primarily low expression of CD25. Only 2-4% of human $CD4^+$ T cells express high levels of CD25, while up to 30% are $CD25^{low}$ or $CD25^{int}$. This staining continuum complicates reliable discrimination between human regulatory and non-regulatory T cells and requires additional analysis of more specific markers [56]. As FOXP3 is the most suitable human T_{Reg} cell marker currently available, its protein expression was examined in MACS separated T_{Reg} cells by FACS during this study. Thereby, clearly more than 80% of the cells were found to express FOXP3 intracellularly. Roncador *et al.* were the first to demonstrate that the majority of human $CD4^+CD25^+$ T cells express FOXP3 and that the frequency of $FOXP3^+$ cells strongly correlates with their CD25 expression level. They found almost 96% of $CD4^+CD25^{high}$ T_{Reg} cells to express FOXP3, while only 35% of the $CD25^{int}$ cells were $FOXP3^+$, indicating that human regulatory T cell activity is mainly enriched within the $CD4^+CD25^{high}$ pool [71]. Compared to these findings, slightly lower FOXP3 frequencies were obtained within the T_{Reg} cell fraction in this work, probably due to staining with a different antibody and to contaminating cells. Since CD25 is currently the most useful marker for identification and isolation, it is more or less impossible to avoid contamination with effector cells [190]. There-

fore, phenotype of these remaining cells was determined and demonstrated to represent mainly natural killer cells and to a lower level, $CD8^+$ T cells, B cells and monocytes. Nevertheless, the isolated $CD4^+CD25^+$ regulatory T cells showed anergic behaviour *in vitro* and well confirmed their suppressive capacity in proliferation assays. These findings indicate that the MACS separated $CD4^+CD25^+$ T cell population was mainly assembled by human $CD4^+CD25^{high}$ T_{Reg} cells. Enrichment of functional and phenotypic human T_{Reg} cells by MACS was further confirmed by the expression profile obtained by application of the *Human T_{Reg} Chip* [Figure 20]. It directly reflected previously described T_{Reg} cell surface markers, such as elevated expression of IL2RA, TFRC, CTLA4 and HLA-DR in the MACS separated $CD4^+CD25^+$ T cells compared to their naive counterparts [Figure 21].

4.2.2 Gene expression signatures of $CD4^+CD25^+$ T_{Reg} cells

cRNA was synthesized from $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells isolated from peripheral blood of 11 healthy donors and hybridized to the *Human T_{Reg} Chip* in order to obtain accurate and reliable individual transcription profiles improving molecular characterization of these T cell fractions. Altogether, 62 genes were identified to be significantly differentially expressed in regulatory compared to naive T cells.

4.2.2.1 FOXP3 affected gene expression Among them, nine genes were revealed that have also been demonstrated to be affected by retroviral over-expression of FOXP3 in $CD4^+$ T_H cells: LGALS3, CCR7, IL2RA (CD25), TNFRSF1B, CTLA4 and TRAF1 were found to be up-regulated in T_{Reg} *versus* naive T cells as well as in FOXP3 transduced $CD4^+$ T_H cells compared to their control cells. In contrast to that, SATB1, GZMK and IL7R gene expression was reduced when comparing the particular cell types in both experiments. Regarding their gene expression profiles, these findings indicate that $CD4^+$ T_H cells infected with the FOXP3 encoding retrovirus do at least partially resemble *ex vivo* isolated T_{Reg} cells. Similarly, a recent microarray study also examined Foxp3-dependent and -independent molecules specific for $CD4^+CD25^+$ naturally occurring T_{Reg} cells, but in the mouse [189]. They could also demonstrate up-regulation of IL2RA and down-regulation of SATB1 in *ex vivo* T_{Reg} *versus* naive T cells as well as in Foxp3 *versus* mock transduced murine T cells thereby confirming the findings of this study and adding data about similarities between mouse and human T_{Reg} cells.

4.2.2.2 Signaling modules in T_{Reg} cells Pathway analysis allowed objective examination of the presented experimental microarray data in the context of known genome-wide interactions by providing more insights into the biological processes and complex signaling networks underlying human T_{Reg} cell immunology. Application of PathwayAssist software to the unique gene expression data set of human regulatory and naive T cells revealed functional modules pointing to mechanisms controlling

survival/apoptosis, T cell receptor signaling/activation/proliferation and differentiation/maintenance of human T_{Reg} cells.

4.2.2.2.1 Genes controlling survival/apoptosis of T_{Reg} cells Naturally occurring T_{Reg} cells survive clonal deletion during their development in the thymus by escape from activation-induced cell death (AICD). This protective mechanism seems to be maintained in T_{Reg} cells encountered in the periphery as a signaling module could be identified that counteracts apoptosis and mediates the release of survival factors [Figure 45].

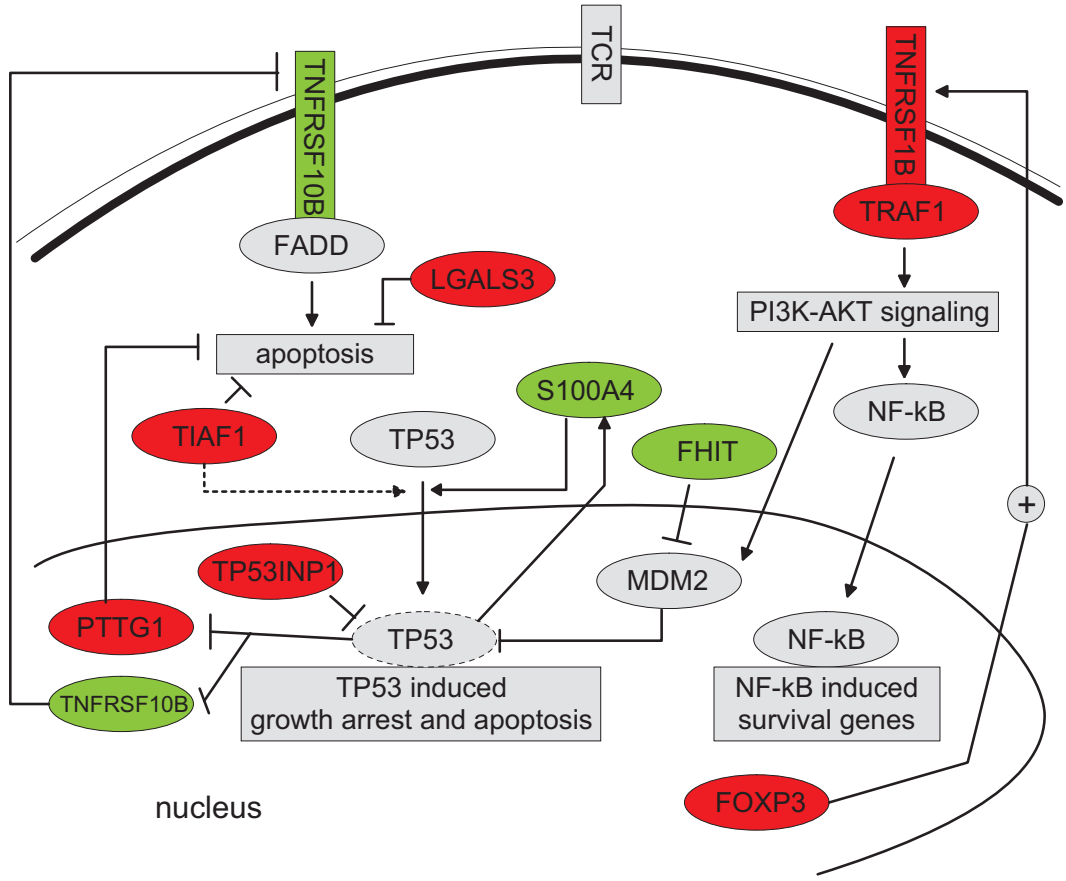

Figure 45: Functional dissection of signaling modules in human T_{Reg} cells. Schematic representation of potential signaling pathways involving genes that control survival/apoptosis of human regulatory T cells thereby mediating T_{Reg} cell functionality. Transcriptional up-regulation of genes in T_{Reg} *versus* naive T cells is marked by red symbols, whereas green symbols represent down-regulated genes. Symbols filled with grey depict unaffected genes or summarize pathway modules.

During this analysis, FOXP3 inducible up-regulation of tumor necrosis factor receptor superfamily member 1B (TNFRSF1B, TNF-RII) was found upon retroviral over-expression in $CD4^+$ $CD25^-$ T cells [Figure 10]. TNFRSF1B was also up-regulated in the *ex vivo* isolated $CD4^+$ $CD25^+$ T_{Reg} cells from individual healthy donors (Figure 20). TNFRSF1B belongs to a group of trans-membrane TNF receptor molecules characterized by TNF receptor-associated factor (TRAF)-interacting motifs (TIMs).

Activation of TIM containing TNF receptors leads to the recruitment of TRAF family members, subsequent activation of signal transduction pathways like NF-κB, JNK, p38, ERK (extracellular signal-related kinase) or PI3K (phosphoinositide 3-kinase), that in turn influence immune responses and increase the expression of survival factors [122, 123]. Well in line, a significant up-regulation of TRAF1 was also revealed in this study in both, FOXP3 transfected $CD4^+CD25^-$ T cells and *ex vivo* isolated human T_{Reg} cells.

This mechanism is linked to additional molecules controlling the nuclear translocation and consequently activity of TP53 (tumor protein p53), a tumor suppressor gene inducing cell growth arrest or apoptosis [124]. While TIAF1 (TGFB-1 induced anti-apoptotic factor 1) interacts with TP53 in the cytosol and may participate in its nuclear translocation, TP53INP1 (TP53 inducible nuclear protein 1) is engaged in the regulation of TP53 activity in the nucleus [125, 126]. Both genes, TP53INP1 and TIAF1 were found to be over-expressed in the naturally occurring T_{Reg} cells in the presented study. Apart from this, TIAF1 is known to be up-regulated in T_H2 *versus* T_H1 lymphocytes and its functional role as an apoptosis protector is discussed [127].

S100A4 was also identified as up-regulated in the naturally occurring T_{Reg} cells from the analysed individual healthy donors. S100A4 is a member of the S100 family of proteins containing 2 EF hand calcium binding motifs. Its expression is TP53 dependent and S100A4 is involved in the regulation of cell cycle progression and differentiation. Together with S100B, S100A4 is hypothesized to control tetramerization of TP53 leading to its nuclear translocation [128, 129]. TP53 can activate the extrinsic apoptotic pathway through the induction of TNF receptor family members, like FAS and TNFRSF10B [124, 130]. Both TNF receptors are characterized by their cytoplasmic death domain which is responsible for transmission of apoptotic signals. Activation of these receptors leads to the recruitment of intracellular death domain containing adaptors such as FAS-associated death domain (FADD) or TNFR associated death domain (TRADD). These molecules activate the caspase cascade and subsequently induce apoptosis. The death domain clearly separates these TNF receptors from TNFRSF1B [122]. As a potential consequence of the assumed TP53 inactivation in T_{Reg} cells TNFRSF10B expression could be impaired.

Further evidence supporting this assumption was provided by another direct target of TP53. PTTG1 expression (pituitary tumor-transforming 1), that was revealed to be up-regulated in the naturally occurring T_{Reg} cells during this study, can be directly repressed by activated TP53 in colorectal cancer cells. RNAi mediated knockdown of PTTG1 was sufficient to induce apoptosis suggesting that repression of novel anti-apoptotic genes by active TP53 can significantly contribute to apoptosis [130]. Controversially, it was reported that PTTG1 can activate TP53 and BAX to increase apoptotic function, but this seems to be rather an indirect effect of PTTG1 and is additionally dependent on other factors, such as MYC, which was found down-regulated in the naturally occurring human T_{Reg} cells [131]. Interestingly, c-MYC is a direct down-

stream target of PTTG1, which is part of the DNA binding complex formed near the transcription initiation site of the c-MYC promoter [132].

During this work, additional genes were detected to be down-regulated in human T_{Reg} cells affecting the activation status of TP53. In lung cancer cells, it was shown that FHIT (fragile histidine triad gene) mediates MDM2 inactivation. The anti-apoptotic molecule MDM2 is activated through the PI3K-AKT pathway leading to inactivation of TP53 [133]. Thus, down-regulation of FHIT also contributes to the inactive status of TP53.

Based on this data, destabilization and thereby inactivation of TP53 may be suggested to provoke a shift in T_{Reg} cells from apoptotic sensitivity to protection and survival. It is tempting to speculate that this mechanism allows T_{Reg} cells to survive upon re-activation, whereas effector T cells underlie activation-induced cell death (AICD). This apoptotic process eliminates the expanded pool of effector lymphocytes during the contraction phase of the immune response and maintains lymphocyte homeostasis. In line with the presented findings, murine T_{Reg} cells were reported to be more resistant to apoptosis when treated with dexamethasone or anti-CD95 Ab than $CD4^+$ total or $CD4^+CD25^-$ effector T cells [134, 135]. Moreover, Fritzsching *et al.* and Wang *et al.* demonstrated that human T_{Reg} cells are less sensitive to activation-induced cell death than their naïve counterparts [136, 104].

Galectin-3 (LGALS3) is one of the best characterized members of the evolutionary conserved family of galectins and was found to be strongly up-regulated in the *ex vivo* isolated T_{Reg} cells [Figure 20]. In addition, LGALS3 was also induced upon FOXP3 over-expression in $CD4^+CD25^-$ T cells [Figure 10]. This is of special interest as LGALS3 is known to participate in apoptosis control. Whereas its secretion triggers apoptotic signal cascades on T cells [137], intracellular expressed LGALS3 acts as an anti-apoptotic molecule [138, 139, 140]. The underlying mechanism was revealed in macrophages, suggesting that LGALS3 may prevent alterations of the mitochondrial membrane and formation of reactive oxygen species. Moreover, it has been reported that LGALS3 phosphorylation is necessary for its anti-apoptotic activity. Taken together, the increased expression level of LGALS3 further supports the idea of a shifted balance towards survival and fitness of T_{Reg} cells.

4.2.2.2.2 Genes controlling TCR signaling, activation and proliferation of T_{Reg} cells The second module that was revealed in this study involves genes controlling T cell receptor (TCR) signaling, activation and proliferation of human T_{Reg} cells [Figure 46].

Galectin-1 (LGALS1) antagonizes T-cell activation by partial phosphorylation of the TCR-ζ chain [141], can block secretion of pro-inflammatory cytokines such as IL2 and skews the balance towards a T_H2-type cytokine profile [142, 143]. Dimeric LGALS1 triggers immunosuppressive IL10 production in T cells contributing to their immune regulatory function [144]. LGALS3 can potentially form complexes on the TCR with

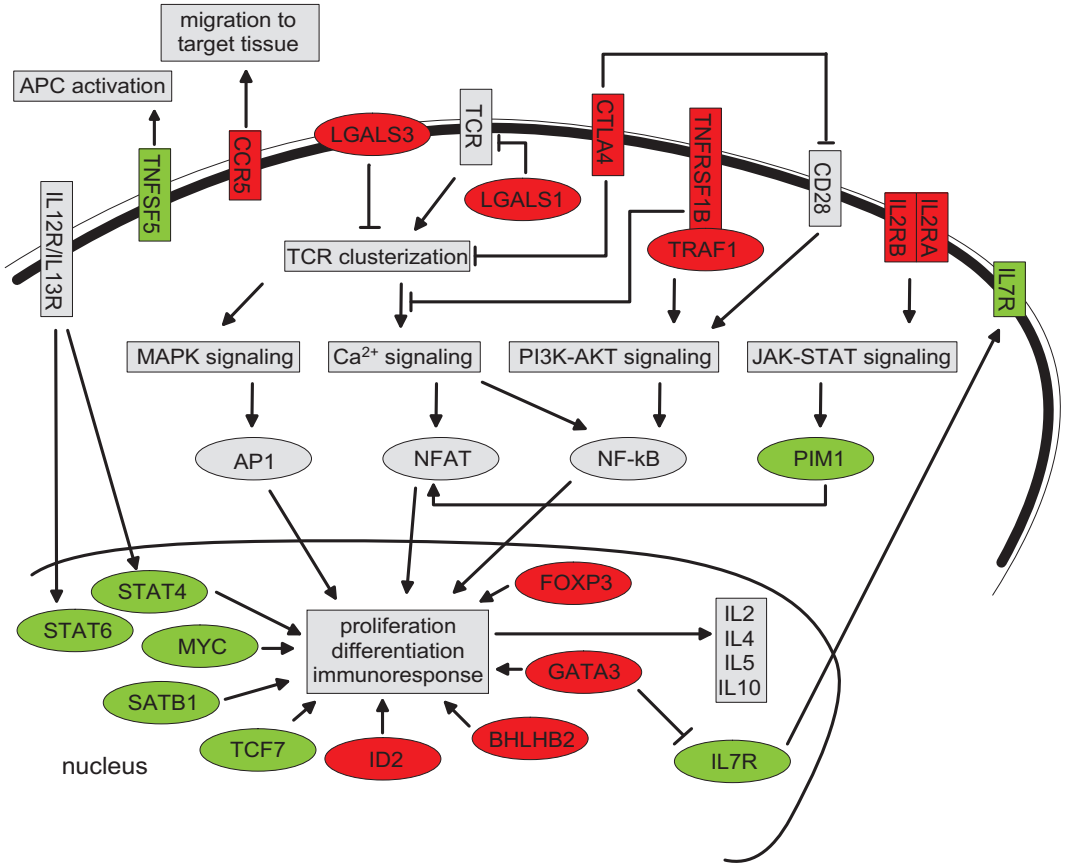

Figure 46: Functional dissection of signaling modules in human T_{Reg} cells. Schematic representation of potential signaling pathways involving genes that control TCR signaling/activation/proliferation and differentiation/maintenance of human regulatory T cells thereby mediating T_{Reg} cell functionality. Transcriptional up-regulation of genes in T_{Reg} *versus* naive T cells is marked by red symbols, whereas green symbols represent down-regulated genes. Symbols filled with grey depict unaffected genes or summarize pathway modules.

N-glycans thereby limiting its lateral mobility resulting in restricted TCR-mediated signaling on T cells [137]. Hence, it may be suggested that up-regulation of both galectins in T_{Reg} cells results in a modulation of their cytokine profile thereby allowing appropriate regulation of effector cells and immune cell homeostasis.

This module also identified a set of genes like CTLA4, TNFRSF1B and PIM1 that controls proliferation within the cell [Figure 46]. CTLA4 plays a major role in inhibiting proliferation of T_{Reg} cells. It is an activation-induced homo-dimeric glycoprotein receptor on T cells interacting with the B7 ligands on the surface of antigen presenting cells (APC). The mechanism of T cell inactivation involves antagonism of CD28-dependent co-stimulation and direct negative signaling through its cytoplasmic tail. When engaged by B7, CTLA4 plays a key role as a negative regulator of T cell activation through down-regulation of cytokine production by preventing the accumulation of AP-1, NF-κB and NFAT in the nucleus. CTLA4 was found to be up-regulated in the human T_{Reg} cells analysed during this work. Its expression has

been linked to enhanced suppressor activity and higher expression of FOXP3 in human T_{Reg} cells. However, the blockade of CTLA4 resulted in a significant but incomplete loss of suppressor activity [145]. In addition to CTLA4, TNFRSF1B was also found to be up-regulated in the isolated human T_{Reg} cells in the presented study. TNFRSF1B is known to be directly co-stimulatory to TCR-mediated activation in human T cells thereby inducing activation markers, like CD25. In contrast to CD28 co-stimulation, cross-linking of TNFRSF1B shows significant differences and triggers different signaling pathways resulting in a modified cytokine profile. TNFRSF1B has the capacity to down-regulate early TCR/CD28 induced Ca^{2+} mobilization and to inhibit T-cell functions such as IL2 and IL10 production [146]. In comparison to activated naïve T cells the proliferation of T_{Reg} cells in response to IL2 is quite low, although the receptor for this cytokine is significantly up-regulated. A serine/threonine kinase called PIM1 could be identified during this project that directly trans-activates NFAT at the end of the Ras signaling cascade to facilitate IL2 dependent proliferation and/or survival of lymphoid cells. Furthermore, PIM1 enhances NFAT-dependent trans-activation and IL2 production in Jurkat T cells [147]. As PIM1 is down-regulated in T_{Reg} cells from individual healthy donors, a reduced signal transmission to NFAT could be proposed mediating less responsiveness to IL2 resulting in lower proliferation of T_{Reg} cells.

4.2.2.2.3 Genes controlling differentiation and maintenance of T_{Reg} cells

A third module extracted by the performed pathway analysis involves genes controlling T_{Reg} cell differentiation and maintenance upon maturation in the thymus [Figure 46]. The differentiation of naïve T cells is induced by TCR activation and either IL12/STAT4 or IL4/STAT6 signaling pathways leading to a T_H1/T_H2 lineage specification that is further directed by the transcription factors T-bet and GATA3, respectively. STAT4 and STAT6 were both down-regulated in the peripheral T_{Reg} cells analysed in the presented study indicating a potential inability to be transformed into T_H cells upon re-stimulation *via* their TCR [Figure 20]. Coexpression of GATA3 and FOXP3, but the lack of T-bet, may point to similarities in the gene expression profiles of T_H2 and T_{Reg} cells in humans.

In a recent study, transcription profiles of T_H1 and T_H2 cells isolated from human cord blood were analysed [150]. Although the overall concordance to the presented T_{Reg}cell data set is quite low, some genes could be detected to be similarly regulated in T_H2 and T_{Reg} *versus* naïve T cells (TCF7, GZMA, S100 family members). However, a few genes showed opposite expression behavior in T_H2 cells compared to the T_{Reg} cells (SATB1 and ACTN1 were up-regulated in T_H2 and down-regulated in T_{Reg} cells). SATB1 and TCF7 are transcription factors functionally similar to GATA3 that have important functions in early thymocyte development [148, 149]. For genes that were differentially expressed in T_H1 *versus* naïve T cells no similarities to the obtained T_{Reg} cell data set could be observed. In summary, these data underline the idea that like their murine homologues, human T_{Reg} cells represent a separate lineage. They

are undergoing a unique differentiation pathway distinct from those committing T_H1 or T_H2 cells and are therefore equipped with a tightly regulated set of transcription factors acting in addition to FOXP3.

Another important question is how T_{Reg} cell populations are regulated and maintained in the periphery. There is growing evidence favoring IL7 as a master regulator of T cell homeostasis, based upon its essential role in the homeostatic expansion of naïve T cells in response to low affinity antigens and its capacity to dramatically enhance expansion of peripheral T cells in response to high affinity antigens [151]. Analyzing a clonal population of mouse $CD4^+CD25^+$ T_{Reg} cells, it was demonstrated that these cells do not proliferate in response to lymphopenia in the absence of the selecting self-peptide. This was in contrast to the naïve T cell proliferation behavior reflecting the lower IL7 receptor (IL7R) expression levels in regulatory compared to naïve T cells [152], which was also supported by the data presented in this study. Additionally, it was shown that GATA3 blocks IL7R expression in early stages of T cell development [153]. As self-antigen presentation in combination with IL7 expression promotes T_{Reg} cell proliferation, it could be assumed that this mechanism contributes to the specific accumulation of T_{Reg} cells at sites where their self-antigen is presented.

4.2.2.3 Implications in T_{Reg} cell mediated diseases Regulatory T cells play a fundamental role in the balance of more or less all immune responses. Thus, developmental or functional deficiencies in T_{Reg} cells could favor disease pathogenesis. Not only modified T_{Reg} cell frequencies, but also gaps in their TCR immune repertoire or genetic polymorphisms leading to aberrant activation, function, survival or homing properties of T_{Reg} cells could be conceivable mechanisms [190]. The vast majority of genes identified as differentially expressed in $CD4^+CD25^+$ regulatory *versus* $CD4^+CD25^-$ naïve T cells constituting the presented fingerprint of human T_{Reg} cells has not yet been described in the context of T_{Reg} cells. As T_{Reg} cells have a far-reaching effect on our health it was investigated, whether these genes have been reported to contribute to pathogenesis in autoimmunity, transplantation tolerance, cancer and even allergy [Table 3]. Strikingly, most of the genes (51 out of 62) were revealed to be implicated in at least one of these disease scenarios underlining their possible importance in function and development of T_{Reg} cells.

4.2.2.3.1 Selected genes involved in autoimmune diseases Autoimmunity occurs as a consequence of self-tolerance break-down presumably resulting from a combination of inherited polymorphisms (or DNA variations), acquired environmental triggers and stochastic events [154]. Analyzing the transcriptional pattern of human T_{Reg} cells isolated from individual healthy donors, 32 genes were revealed to be involved in the pathogenesis of diverse autoimmune diseases [Table 3]. The following discussion will focus only on a few affected genes that are central of the functional modules described above and that might therefore influence disease pathogenesis.

For example, TNFRSF1B was shown to be 2.5-fold over-expressed in the naturally occurring T_{Reg} cells compared to their naïve counterparts. A single nucleotide polymorphism (SNP) in this gene was reported to influence susceptibility to multiple sclerosis, a severe inflammatory autoimmune disorder of the central nervous system [81]. In addition, Sashio *et al.* linked two other polymorphisms to the TNFRSF1B gene locus that increase susceptibility to Crohn's disease and ulcerative colitis, which are both chronic inflammatory diseases of the gastrointestinal tract [82]. In Japanese patients, Morita *et al.* identified another SNP in the TNFRSF1B gene associated with systemic lupus erythematosus (SLE) , a chronic, relapsing, inflammatory and often febrile multisystemic disorder of autoimmune origin [155].

Type I diabetes is a T-cell mediated inflammatory autoimmune disease of the endocrine pancreas, resulting in lack of insulin caused by beta cell destruction. Altogether, 18 genes were included in the presented human T_{Reg} cell signature that have been reported to contribute to pathogenesis of this disease, e.g. granzyme A (GZMA) [156], the CD40 ligand (TNFSF5) [157, 158], CTLA4 [83] and the T-cell specific transcription factor 7 (TCF7) [159]. Furthermore, two polymorphisms in the HLA-DRB1 gene, which was found over-expressed in T_{Reg} cells, have been described to confer high-risk susceptibility [160].

Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting the joints probably caused by autoimmune mechanisms. 21 T_{Reg} cell specific genes revealed in this study have been described as susceptibility genes for RA. For example, LGALS3 [161, 162], GZMA [163] and the S100 calcium binding protein A4 (S100A4) [164] were described as highly expressed in the synovial tissue and at sites of joint destruction contributing to the inflammatory process. The complex genetic component of RA etiology was further demonstrated by the discovery of multiple polymorphisms, for example in genes of the chemokine receptor 5 (CCR5) [84] and of HLA-DRB1 [165], conferring high risk susceptibility.

In mice deficient for signal transducer and activator of transcription 4 (STAT4), a gene also found to be down-regulated in the analysed human T_{Reg} cells, RA is suppressed due to reduced levels of IL12 and INF-γ [166]. Interestingly, $STAT4^{-/-}$ mice were additionally almost completely protected from diabetes [167] and the induction of experimental allergic encephalomyelitis [168] underlining the importance of STAT4 down-regulation in T_{Reg} cells for their proper immune regulatory function.

As T_{Reg} cells are essential for the maintenance of self-tolerance, SNPs or mutations affecting genes expressed in T_{Reg} cells may result in the synthesis of aberrant mRNAs and proteins which in turn could impair T_{Reg} cell function and/or development leading to higher risks for autoimmunity. Additionally, failures in gene regulation resulting in inadequate protein amounts could disturb appropriate T_{Reg} cell activity thereby most likely contributing to the pathogenesis of autoimmune disorders.

As most of the genes discussed here, are central components of the functional modules discussed in chapter 4.2.2.2, it is conceivable that the dys-regulation of one or

more of these genes affect T_{Reg} cell action in terms of survival/apoptosis, differentiation, proliferation and suppressor function thereby promoting break-down of self-tolerance eventually leading to autoimmunity. However, further research is needed in order to better understand the complex mechanisms of genetic background and environmental factors that control the development of autoimmunity.

4.2.2.3.2 Selected genes involved in cancer T_{Reg} cells are characterized by their ability to suppress host immune responses and to induce self-tolerance. At tumor sites, antigen-specific T_{Reg} cells are supposed to attenuate anti-tumor immunity [169, 170, 171, 172]. Elevated frequencies of $CD4^+CD25^+$ T_{Reg} cells with functional suppressive activity were recently reported in tumors and blood of patients suffering from different human cancers, among them lung, breast and ovarian tumors [86, 90, 91], hepatocellular [40] and squamous cell head and neck carcinoma [39] as well as in chronic lymphocytic leukemia [41]. Furthermore, elimination of T_{Reg} cells in mouse models enhanced anti-tumor responses [173, 174, 170]. In addition to the T_{Reg} cell mediated immunosuppressive microenvironment, the tumor itself can escape from immune surveillance by counteracting apoptotic signals delivered by activated immune cells. Strikingly, a high degree of concordance in gene expression could be observed during data analysis contributing to both T_{Reg} cell activity and tumor survival.

44 out of the 62 specific T_{Reg} cell genes reported in this study are also expressed by tumor cells thereby participating in cancer development and progression [Table 3]. Among them, one can find several tumor suppressors (like STAT4, FHIT and TNFRSF10B) as well as proto-oncogenes (e.g. PIM1, MYC, XBP1 and PTTG1): For example, aberrant transcripts or complete lack of the fragile histidine triad gene FHIT were observed in esophageal, stomach, colon [175] and clear cell renal carcinomas [176] as well as in lung [177], head and neck [178] and cervical cancer [179]. Furthermore, inactivation of one or both Fhit alleles in mice dramatically increased development of multiple tumors, underlining the importance of FHIT as a tumor suppressor gene [180]. Down-regulated FHIT expression was found in human T_{Reg} cells in this study, while the pituitary tumor-transforming 1 gene (PTTG1) was revealed to be over-expressed in T_{Reg} cells [Figure 20]. High PTTG1 expression levels have been reported in various human tumors, including pituitary, adrenal, kidney, liver and ovarian cancer resulting from enhanced cell proliferation and cellular transformation [181].

Furthermore, it could be demonstrated that LGALS3, which is a member of the β-galactoside-binding gene family, was highly up-regulated in regulatory *versus* naïve T cells and was induced upon retroviral over-expression of FOXP3 in $CD4^+CD25^-$ T cells. This multifunctional protein has been implicated in tumor cell adhesion, proliferation, differentiation, angiogenesis, cancer progression and metastasis [138]. Intracellular LGALS3 exhibits anti-apoptotic activity thus contributing to cell survival [139, 140]. Conversely, extracellular LGALS3 was additionally shown to be secreted by tumor cells themselves in order to induce apoptosis of cancer-infiltrating cells thereby supporting tumor immune escape [137].

As reflected by their co-regulated gene repertoire, both T_{Reg} and tumor cells may employ similar mechanisms to mediate immune suppression in their particular microenvironment. Activated T cells directed against the tumor or against self-antigens are controlled by both cell types either to escape from immune surveillance or to protect from autoimmunity. Furthermore, increasing evidence argues that up-regulation of T_{Reg} cell frequencies observed in cancer patients is tumor driven [172]. This supports the idea of partly concordant pathways leading to the induction of tolerance towards antigens encountered under diverse clinical conditions ranging from autoimmunity to cancer.

4.2.2.3.3 Selected genes involved in transplantation tolerance As part of their immune suppressive function, T_{Reg} cells are able to induce transplantation tolerance thereby contributing to graft survival [44, 42]. Out of the 62 T_{Reg} cell specific genes, 20 candidates have been described to influence graft fate [Table 3]. CCR5 up-regulation was shown in T_{Reg} cells in the presented work, which is concordant with recent results demonstrating that CCR5 is essential for preventing graft-*versus*-host disease (GvHD) in murine models. In their study, Wysocki *et. al* demonstrated that CCR5 is not only guiding T_{Reg} cells to lymphoid tissues in order to down-regulate effector cell populations, but also allows their migration to GvHD target tissues to improve graft survival [96]. Another gene found over-expressed in the analysed T_{Reg} cells is the TGFB1-induced anti-apoptotic factor 1 (TIAF1). It was reported that TIAF1 expression in lymphocytes during chronic kidney and liver allograft rejection may protect these cells from apoptosis [127]. Furthermore, down-regulated expression of interleukin 7 receptor (IL7R) and chemokine (C-C motif) receptor 7 (CCR7) could be demonstrated in this study. CCR7 is a lymphoid-specific member of the G protein-coupled receptor family that together with its ligands regulates T cell migration to secondary lymphoid organs [182]. $CCR7^{-/-}$ mice completely failed to reject MHC class I mismatched grafts implicating its central role in transplant rejection [183]. Antagonists of CCR7 ligands were additionally demonstrated to provide beneficial effects for the prevention of chronic GvHD in mice by blocking the homing of donor CCR7-expressing T cells into the recipients lymphoid organs [184]. Furthermore, it was shown, that binding of IL7 to its receptor enhances peripheral T cell reconstitution after allogeneic hematopoietic stem cell transplantation by selectively mediating proliferation and anti-apoptotic effects to non-alloreactive and de novo-generated T cells [185].

Taken together, the genes reported here to affect transplantation tolerance are important mediators of a balanced immune response by controlling migration, apoptosis and survival of T cells.

4.2.2.3.4 Selected genes implicated in allergy Allergic hyper-reactivity is characterized by an exaggerated immune response towards harmless antigens caused by dysregulated responsiveness of T_H2 cells [99]. During the allergic immune response

high levels of the T_H2 cytokines IL4, IL5 and IL15 are produced, leading to secretion of allergen-specific IgE and recruitment of eosinophils [190]. The ability of regulatory T cells to suppress T_H2 cytokine production suggests that T_{Reg} cells play an important part in the development of tolerance to allergens [100]. Out of the 62 genes constituting the presented T_{Reg} cell expression profile, 19 candidates have been described to be implicated in allergic disease pathogenesis, including such diverse disorders like e.g. asthma, psoriasis or celiac disease [Table 3].

For example, the G protein-coupled receptor 2 (GPR2 or CCR10) is known as a skin-homing receptor and its up-regulation and interaction with its ligand CCL27 was demonstrated in skin lesions of atopic dermatitis, contact dermatitis and psoriasis patients [186]. CCR10 was found to be over-expressed in human T_{Reg} cells compared to their naive counterparts isolated from healthy donors during this work.

The chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES), which was revealed to be down-regulated in regulatory *versus* naïve human T cells, was shown to play a crucial role as a mediator in the immuno-pathogenesis of house dust mite-induced chronic bronchitis. Increasing CCL5 serum levels correlated with disease progression from stable *via* chronic bronchitis to asthma [187]. Furthermore a polymorphism in the RANTES promoter was associated with sensitization to cat and mold allergens [250], underlining the importance of CCL5 for adequate T_{Reg} cell function.

4.2.3 More news about the phenotype of human regulatory T cells

Quantitative real-time RT-PCR on selected transcripts was performed as a sensitive independent verification method of the presented microarray results. Therefore, well-accepted T_{Reg} cell specific genes have been chosen, such as FOXP3, CTLA4 and CCR7, as well as five newly revealed candidate genes (LGALS3, TNFRSF1B, CCR10, CCR5, TRAF1). For all these selected genes a high correlation between PCR and microarray results could be demonstrate thereby lending confidence to the creditability of the applied microarray approach and suggesting a possible role of the 41 "new players" in human T_{Reg} cell activity. Observed quantitative differences in the fold changes have been described before; especially an underestimation of real expression changes by microarray approach *versus* quantitative RT-PCR has been reported [108, 121] and is thought to result at least partly from the applied normalization procedure. Besides validating the microarray data, quantitative RT-PCR also confirmed the applied cell separation strategy yielding highly pure regulatory as well as naive T cells. As described above, FOXP3 is crucial for development and function of $CD4^+$ $CD25^+$ T_{Reg} cells and is generally accepted as their most specific molecular marker available to date [52]. FOXP3 was found to be up-regulated 2.5fold by microarray and 11.6fold by qRT-PCR in regulatory compared to naive T cells, thereby underlining the regulatory phenotype of the isolated $CD4^+CD25^+$ T_{Reg} cells. The observed fold changes for FOXP3 expression are similar to those described previously for $CD4^+CD25^{high}$ T_{Reg} cells *versus* $CD4^+CD25^-$ naive T cells isolated by MACS technology from peripheral blood of healthy donors [41, 234].

After confirming elevated gene expression of LGALS3, CCR10, CCR5, TRAF1 and TNFRSF1B on mRNA level in human $CD4^+CD25^{high}$ T_{Reg} *versus* $CD4^+CD25^-$ naive T cells, three candidates were selected to verify their up-regulation on protein level. LGALS3 was chosen because it showed highest over-expression in T_{Reg} cells among the 41 new candidate genes revealed by microarray analyses. TNFRSF1B and CCR10 were selected for their possible qualification as convenient T_{Reg} cell markers as both proteins are expressed at the cell surface. Applying FACS analysis, statistically significant higher amounts of LGALS3 and TNFRSF1B protein expression could be confirmed in human $CD4^+CD25^{high}$ T_{Reg} cells compared to $CD4^+CD25^-$ T cells of healthy donors. Compared to FOXP3 as the currently most accepted T_{Reg} cell marker, both proteins were expressed by a similar fraction of $CD4^+$ T cells. To our knowledge, this is the first study demonstrating LGALS3 and TNFRSF1B up-regulation on mRNA and protein level in human peripheral blood $CD4^+CD25^{high}$ T_{Reg} cells compared to their naive counterparts [196, 197]. Only one study investigating human $CD4^+CD25^+$ T cells purified from postnatal thymuses assessed TNFRSF1B gene and protein expression. They found that almost all of these $CD4^+CD25^+$ thymocytes constitutively over-expressed TNFRSF1B mRNA and protein compared to $CD4^+CD25^-$ T cells. They explain differences in gene/protein expression between peripheral blood and thymus-derived human T_{Reg} cells by the fact that $CD4^+CD25^+$ thymocytes represent a more homogenous population [194]. Again consistent with the findings presented here, another study on mouse T_{Reg} cells reported TNFRSF1B up-regulation on mRNA level in regulatory *versus* naive T cells.

It is now widely accepted, that natural T_{Reg} cells are positively selected in the thymic cortex through their TCR interactions with self-peptides presented by thymic stromal cells. Probably during this high-affinity recognition T_{Reg} cells receive signals rendering them anergic and able to produce anti-apoptotic molecules which protect them from apoptosis [194]. The results presented here in this study may suggest that TNFRSF1B is one of these protective molecules as signals derived from this receptor inhibit TCR-induced apoptosis and prolong survival [122]. Furthermore, TNFRSF1B induction seems to be an important process in thymic T_{Reg} cell maturation as its over-expression is maintained in mouse and human T_{Reg} cells entering the periphery. This hypothesis is further supported by the shown up-regulation of LGALS3 gene and protein expression in human peripheral blood T_{Reg} cells. Galectin-3 is the only member in its galectin family that possesses anti-apoptotic abilities when expressed intracellularly [139, 138]. LGALS3 was shown to protect T cells, macrophages and various carcinoma cells from cell death triggered by a variety of agents [195]. As discussed in more detail before (chapter 4.2.2.2.1), the performed analyses revealed even more molecules involved in apoptosis control which were summarized in a functional module counteracting apoptosis. Combined Annexin and PI staining followed by subsequent FACS analysis indeed confirmed that human T_{Reg} cells are less susceptible towards apoptotic cell death than their naive counterparts thereby supporting the idea of a

shifted balance towards survival and fitness of T_{Reg} cells. Well in line with these findings is a mouse study investigating the thymic selection of $CD25^+$ T_{Reg} cells. It points out that regulatory T cells seem to be more resistant to clonal deletion than their naive counterparts resulting in selective survival [191].

Next, coexpression of LGALS3 and TNFRSF1B, respectively, with FOXP3 protein was confirmed on a small fraction of $CD4^+$ T cells and revealed an even more extended population that was just single positive for either LGALS3 or TNFRSF1B but expressed no FOXP3 protein. Furthermore, a fraction of $CD4^+$ cells could be indentified expressing FOXP3 protein but lacking expression of LGALS3 and TNFRSF1B, respectively. Expression of LGALS3 has been observed in different cell types and tissues, among them monocytes and macrophages, DCs, mast cells, granulocytes and murine T cells [135]. Furthermore, TNFRSF1B expression was shown in resting and activated human T cells [122] thereby also supporting the obtained findings. Whether the various subpopulations identified differ in their suppressive activity remains to be elucidated. For example, it has been reported that among $CD4^+CD25^+$ T cells those expressing CD103 (ITGAE) or CD62L are more potent suppressors than their negative counterparts [69]. Within the $CD4^+CD25^+$ T cell subset, CD27, that like TNFRSF1B belongs to the TNFR family, was demonstrated to discriminate regulatory from activated effector T cells. The highest frequency of $FOXP3^+$ cells was thereby found in the $CD4^+CD25^+CD27^+$ subset, but was only 42%. Nevertheless, suppressive activity was restricted to this $CD4^+CD25^+CD27^+$ subpopulation. In contrast to that, $CD4^+CD25^+CD27^-$ T cells still contained 6.2% $FOXP3^+$ cells but did not influence proliferation of responder cells at all [72]. Thus, FOXP3 can also be induced in conventional T cells upon activation showing no suppressive capacity [16, 73]. Hence, it is tempting to speculate that apart from FOXP3, other molecules or more probably combinations of them might be able to confer regulatory capacities. In contrast to results obtained from mice, human T_{Reg} cell biology seems to be still more complex and diverse.

As availability of specific marker molecules is still limited, isolation and hence characterization of human T_{Reg} cells remain difficult. In the mouse, CD25 is a good marker for T_{Reg} cells, as animals are held under pathogen-free conditions. In contrast to that, humans are constantly exposed to foreign antigens, leading to a significant fraction of recently activated non-regulatory $CD25^+$ effector T cells. During the search for more specific T_{Reg} cell markers, FOXP3 has been identified as uniquely expressed in mouse T_{Reg} cells and has emerged as a lineage marker in developing mouse T_{Reg} cells. As expected and previously reported, *ex vivo* human $CD4^+CD25^+$ T cells isolated in this study expressed significantly higher amounts of both FOXP3 mRNA and protein than did $CD4^+CD25^-$ T cells. Furthermore, it was demonstrated that FOXP3 mRNA was consistently induced after 3 days of activation with anti-CD3 $\pm$ IL2 even in non-regulatory $CD4^+CD25^-$ T cells, and FOXP3 protein was clearly detectable after 5 days. However, FOXP3 mRNA and protein levels never reached those observed in $CD4^+CD25^+$ T_{Reg} cells. The capacity of human $CD4^+CD25^-$ T cells to up-regulate

FOXP3 gene and protein expression following activation and expansion has recently been described [16, 73] thereby supporting the presented results. Allan *et al.* further demonstrated that retroviral over-expression of FOXP3 in human $CD4^+CD25^-$ T cells alone is not sufficient to generate potent suppressor T cells *in vitro* that are functionally equivalent to $CD4^+CD25^+$ T_{Reg} cells [16]. Although FOXP3 is clearly a link in the regulatory circuit controlling immunological tolerance, it does not appear to be the sole master switch. These experiments rather suggest additional factors required during the process of activation and/or differentiation finally resulting in the development of *bona fide* human T_{Reg} cells [52]. A number of studies have proposed additional molecules that are expressed at higher levels on $CD4^+CD25^+$ T_{Reg} compared to naive T cells. Among them, inhibitory costimulatory receptors like PD1, GITR, TNFRSF9 and TNFRSF4, as well as chemokine (CCR4), toll-like (TLR4) or homing receptors (CD103) have been described. Recently discussed putative marker molecules are LAG-3, Nrp1 and CD27. However, the majority of surface proteins are also up-regulated on $CD4^+CD25^-$ T cells upon activation while several markers were just found to be of importance in mouse but not human T_{Reg} cells [190].

To further assess qualification of LGALS3 and TNFRSF1B as putative new T_{Reg} cell markers, *in vitro* activation experiments were performed. Thereby, constitutive and high expression of both genes could be confirmed on mRNA and protein level in activated human $CD4^+CD25^+$ T_{Reg} cells. Little induction of TNFRSF1B mRNA was observed in activated $CD4^+CD25^-$ T cells, but expression levels remained clearly lower than those in activated and *ex vivo* T_{Reg} cells thereby showing similar expression behaviour like FOXP3. The only study comparing TNFRSF1B mRNA levels between recently activated and *ex vivo* isolated $CD4^+CD25^-$ T cells was carried out by Gavin and co-workers in the mouse. In contrast to results presented here, they reported a slight decrease in TNFRSF1B mRNA expression, which is probably due to a different activation strategy (ConA instead of anti-CD3; activation for 46h compared to 3 days), but may also point to interesting interspecies differences [190]. LGALS3 mRNA was so strongly induced in $CD4^+CD25^-$ T cells upon activation that expression levels exceeded that of *ex vivo* T_{Reg} cells. But LGALS3 mRNA expression in activated effector T cells still remained clearly lower than that observed in activated T_{Reg} cells, as LGALS3 mRNA expression was elevated most of all upon activation in T_{Reg} cells. FACS analysis of LGALS3 and TNFRSF1B protein levels revealed that expression of both proteins was highly up-regulated in activated $CD4^+CD25^+$ T_{Reg} cells compared to $CD4^+CD25^-$ effector T cells upon activation. The shift in LGALS3 and TNFRSF1B expression between both subpopulation turned out to be even more pronounced than that observed for *ex vivo* cells. As LGALS3 protein expression is restricted at low levels to nonactivated human peripheral blood $CD4^+CD25^+$ cells, a high level of LGALS3 protein represents a T_{Reg} specific FOXP3-signature of antigen-stimulated human $CD4^+CD25^{high}$ derived T_{Reg} cells [197]. Taken together, these findings suggest LGALS3 and TNFRSF1B as suitable new T_{Reg} cell marker molecules in healthy humans that could facilitate T_{Reg} cell isolation and thus improve T_{Reg} cell characterization in future.

4.3 Regulatory T cells in RCC

4.3.1 Elevated T_{Reg} cell frequency

The emergence of a tumor results from the disruption of cell growth regulation as well as from failure of the host to provoke a sufficient immunological anti-tumor response. Several tumor-specific immune evasion strategies have been described, among them insufficient presentation of T cell activating epitopes through down-regulation of MHC class I expression and other proteins involved in processing antigenic peptides, secretion of tumor-derived immunosuppressive and anti-apoptotic molecules or induction of immunotolerance against tumor-specific antigens [85]. Accumulation of T_{Reg} cells in peripheral blood and tumor environment of cancer patients was reported by numerous investigators and is thought to represent an additional mechanism promoting tumor immune escape [41, 85]. Based on these observations it was interesting to examine whether T_{Reg} cell frequencies are also increased in peripheral blood of RCC patients and, if so, whether T_{Reg} cell numbers correlate with stage of disease. Indeed, a significantly elevated presence of $CD4^+CD25^{high}$ T_{Reg} cells could be observed in peripheral blood of RCC patients compared to healthy controls in this study, which was accompanied by a significant increase in total numbers of $CD4^+CD25^+$ T cells. Two studies published during this work supported these findings: Cesana *et al.* also reported elevated frequencies of otherwise functionally suppressive $CD4^+CD25^{high}$ T cells in PB of metastatic RCC patients and even percentages given were very similar to those observed in the presented work. Furthermore they discovered an increase in T_{Reg} cell numbers following IL2 therapy which remained elevated in patients with disease progression, but returned to normal levels in patients with objective clinical response [198]. Similarly, Dannull *et al.* measured higher T_{Reg} cell frequencies in peripheral blood of metastatic RCC patients when compared to healthy controls. Furthermore, they demonstrated that selective elimination of T_{Reg} cells followed by vaccination with tumor RNA-transfected DCs significantly improved stimulation of tumor-specific T cell responses in RCC patients when compared with vaccination alone [95]. Thus, increased frequency of T_{Reg} cells with functional suppressive activity may have potential implications for the treatment of cancer patients and might explain the poor clinical response of cancer patients undergoing immunotherapy. Depletion of T_{Reg} cells in combination with tumor immunotherapy or vaccination could have the potential to enhance chances of RCC eradication. Increasing evidence suggests that elevated T_{Reg} cell numbers could at least in part be tumor-driven [63], as e.g. removal of HCC tumors led to restoration of normal T_{Reg} cell frequencies [40]. Furthermore, T_{Reg} cell frequency was compared between metastatic and localized tumor growth in PB of RCC patients but no statistically significant differences could be uncovered. This is well in line with a previous study reporting no substantial change in T_{Reg} cell numbers among AML subtypes [104].

4.3.2 Gene expression signatures of $CD4^+CD25^+$ T_{Reg} cells in RCC

Despite recent advances in human T_{Reg} cell research, the molecular and cellular mechanisms responsible for the increase and maintenance of elevated levels of T_{Reg} cells in cancer remain elusive. Furthermore, little is known about potential changes in T_{Reg} cell development and/or function during tumor growth leading to augmented suppression that may facilitate tumor immune escape [63]. Along with other solid malignancies, RCC continues to progress despite significant numbers of tumor-infiltrating lymphocytes (TIL), implying potential host immune dysfunction and poor tumor antigen recognition and/or presentation [199]. As microarrays have demonstrated their potential to unravel gene expression of various subsets of leukocytes [200], this technology was applied in the presented study to reveal gene expression signatures of human regulatory T cells. A comparison between transcriptional patterns of T_{Reg} cells obtained either from healthy donors or RCC patients may contribute to a better understanding of the molecular mechanisms underlying T_{Reg} cell mediated tolerance in health and cancer.

Comparative microarray analyses of $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells revealed 81 genes significantly differentially expressed between both T cell subpopulation in RCC patients, while only 62 genes were regulated in healthy donors. The 81 candidate genes in RCC were composed of 49 over-expressed and 32 down-regulated genes when comparing T_{Reg} to naive T cells (healthy: 32↑ *versus* 30↓). Separated examination of both T cell subsets in either localized or metastatic RCC still more clearly underlined that the majority of genes identified in RCC showed over-expression in T_{Reg} cells (localized: 43↑ *versus* 29↓; metastatic: 63↑ *versus* 22↓). Thus, a rising number of regulated genes was identified comparing healthy donors *via* localized to metastatic RCC (n=62, 72, 85) and thereby especially the fraction of candidates over-expressed in T_{Reg} cells increased dramatically depending on stage of disease (n=32, 43, 63). These findings may point to a gain-of-function in T_{Reg} cells from RCC patients that could equip them with additional properties and/or survival strategies (compared to those in healthy donors) supporting tumor immune protection.

Many of the genes identified as differentially expressed between $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells in localized and metastatic RCC could be linked to participate in apoptosis control. Among them, one can find the galectins LGALS1 and LGALS3 again, which were already revealed when analysing healthy donors. LGALS1 was demonstrated to be able to induce apoptosis in activated T cells and was shown to be expressed by various, especially aggressive and metastatic tumor cells. Thus, secretion of LGASL1 by the tumor itself could induce apoptosis in otherwise anti-tumor directed, cytotoxic T cells and may be supposed as a possible mechanism of tumor immune escape [138, 139, 140]. Blocking of LGALS1 in a tumor mouse model resulted in an increased tumor-specific T cell response *in vivo* and even led to reduction in tumor size [138], thereby supporting this idea. As LGALS1 expression was also elevated in T_{Reg} cells isolated from RCC patients compared to healthy controls (+3.4),

an augmented ability of RCC T_{Reg} cells to render tumorantigen-specific T effector cells apoptotic could be supposed, hence facilitating tumor growth. This hypothesis is supported by the fact, that RCC continues to progress despite the presence of significant numbers of tumor-infiltrating lymphocytes [199], thus procedures (like apoptosis induction by the tumor itself or by T_{Reg} cells) seem to exist, that counteract tumor combat. Furthermore, recent studies have re-examined T_{Reg} cell induced apoptosis of effector T cells as a potential mechanism for suppression. Grossman *et al.* reported that human natural and adaptive T_{Reg} cells mediate their suppressive effects by a GZMA/perforin and ITGB2-dependent pathway to cause autologous target cell death [201]. Similarly, Parakishvili *et al.* demonstrated that $CD4^+$/perforin$^+$ T cells from B-CLL patients are able to kill autologous B-CLL cells *ex vivo via* a perforin-mediated mechanism [203]. More recent studies have shown that T_{Reg} cells even suppress cytokine secretion, proliferation, and cytotoxic activity of anti-tumor NK cells, which have been reported to be important cells for tumor clearance [172].

LGALS3 over-expression was higher in T_{Reg} cells compared to their naive counterparts in healthy individuals (+3.9, microarray analysis) *versus* RCC patients (+1.7) and showed slightly increased expression in RCC T_{Reg} cells compared to T_{Reg} cells of healthy volunteers (+2.2). These results are confirmed by a microarray analysis examining PBMCs from patients with advanced RCC, that also revealed up-regulation of LGALS3 compared to the healthy control group and even incorporated this molecule in a set of eight predictor genes, definitely distinguishing PBMCs from RCC patients and healthy donors [202]. As described previously, LGALS3 may play distinct roles in apoptosis control depending on its localization. Extracellularily secreted LGALS3 is functioning as a pro-apoptotic protein [138], thus elevated levels in RCC T_{Reg} cells may in concert with LGALS1 induce apoptotic cell death in tumor-specific effector T cells, hence contributing to tumor immune escape. In contrast to that, intracellularly expressed LGALS3 was reported to trigger anti-apoptotic pathways in T cells [138], thereby promoting T cell survival and providing a possible explanation approach for the increased frequency of T_{Reg} cells in RCC.

BAX (BCL2-associated X protein) belongs to the BCL2 protein family, representing a critical intracellular checkpoint in the apoptotic pathway. BCL2 family members form hetero- or homodimers and act as pro- or anti-apoptotic regulators. As an apoptotic activator, BAX is reported to interact with, and increase the opening of mitochondrial anion channels, which leads to a loss in membrane potential. Release of cytochrome c and subsequent caspase activation finally results in programmed cell death. BAX forms heterodimers with BCL2, that possesses apoptotic repressor activity and is thus functioning as a BAX antagonist. Expression of both, BAX and BCL2 genes, were shown to be regulated by the tumor suppressor P53 [205]. Furthermore, sensitivity of RCC cell lines to INF-α has been correlated with p53-induction and involved its downstream target BAX [204]. Interestingly, a marked down-regulation of BAX expression was identified in T_{Reg} cells of RCC patients compared to healthy donors (-3.4) during

this work which was accompanied by an up-regulation of its repressor BCL2 (+2.4) in T_{Reg} cells *versus* naive T cells of metastatic RCC patients. Over-expression of the BCL2 oncogene has been demonstrated to increase the resistance of lymphocytes to many different death stimuli, including cytokine withdrawal, DNA damage and oxidative stress [205]. Thus, the results presented here provide further evidence for a protection from apoptosis and thus an enhanced survival of T_{Reg} cells in RCC contributing to their accumulation.

Interleukin-7 (IL7) plays an important role in the normal development and maintenance of the human immune system. Engagement to the IL7 receptor (IL7R) initiates a cascade of phosphorylation events triggered by various molecules including Janus kinases (JAK1 und JAK3), PI3 kinase and STATs. Activation of IL7 signalling pathway finally results in survival, proliferation, differentiation and maturation of haematopoietic cells including T lymphocytes. Elevated levels of IL7 and IL7R expression were correlated with genesis of especially aggressive malignancies with poor prognosis, such as certain types of leukemia, lymphomas and breast cancer [211]. Interestingly, knockout studies in mice suggested that blocking apoptosis is an essential function of IL7R during differentiation and activation of T lymphocytes [208]. Although T cell development could be observed in IL7R-deficient animals, the number of thymocytes was significantly reduced, implying a role of IL7R in controlling the size of the thymic T cell compartment. Inversely, over-expression of IL7R in mice was shown to result in increased survival of thymocytes due to decreased apoptosis rates [212]. Strikingly, opposite regulation of IL7R was observed in T_{Reg} cells from healthy donors and RCC patients in this study. While T_{Reg} cells of healthy volunteers expressed significantly lower amounts of IL7R than their naive counterparts (-2.1), up-regulation of IL7R expression was detected in T_{Reg} *versus* naive T cells from metastatic RCC patients (+1.6) and when comparing RCC *versus* healthy T_{Reg} cells (+2.6). A recent publication confirms these results by reporting inverse correlation between IL7R and FOXP3 expression as well as between IL7R and suppressive function in human T_{Reg} cells [213]. Once more, these findings support the theory of enhanced survival due to decreased apoptosis as a mechanism to elevate T_{Reg} cell frequencies in RCC.

Members of the TNF-α superfamily are type I membrane-bound cell surface polypeptides that signal to target cells upon cell-cell contact or after protease-mediated release to the extracellular space. Four members of this family - FASL, TNF-α, TL1A and TRAIL - are able to induce cell death under particular circumstances. Binding of ligand to the extracellular domain of its receptor, enhances the affinity of the intracellular death domain for the cytoplasmic adaptor protein FADD. FADD binding results in activation of a caspase cascade through both mitochondria-dependent and -independent pathways that finally end up in apoptosis. Several TRAIL receptors (TRAILR) exist: TRAILR1 is also called death receptor 4 or TNFRSF10A, TRAILR2 is also known as DR5 or TNFRSF10B. Interestingly, DR4 expression was discovered to be markedly down-regulated in T_{Reg} cells from RCC patients compared to healthy controls (-3.3)

and expression of TNFRSF10C as a third, but decoy TRAILR was clearly decreased in T_{Reg} *versus* naive T cells from RCC patients (-3.0) with an even more significant decline in metastatic RCC patients (-4.1). Again, these findings may point to a decreased susceptibility of RCC T_{Reg} cells towards apoptosis. Well in line, a recent study demonstrated selective resistance of naturally occurring human $CD4^+CD25^+FOXP3^+$ T_{Reg} cells to rapamycin-induced apoptosis [206]. Concordant results were obtained by a study examining T_{Reg} cells in acute myeloid leukemia (AML) patients. It could be demonstrated that the fraction of apoptotic cells within the $CD4^+CD25^{high}$ T_{Reg} cells was the lowest when compared to the remaining $CD4^+$ T cell subsets ($CD25^{low}$, $CD25^-$). But compared to healthy donors, an elevated frequency of apoptotic cells was observed within the $CD4^+CD25^{high}$ T_{Reg} cell population of AML patients. Authors could show, that this effect was compensated by an increased proliferation of AML T_{Reg} cells compared to healthy donors [104].

Thus, not only diminished susceptibility towards apoptosis but additionally increased proliferation rates in T_{Reg} cells from RCC donors could be responsible for higher T_{Reg} cell frequencies in RCC patients. Expression profiling of T_{Reg} and naive T cells from healthy donors as well as from patients suffering from localized or metastatic RCC indeed identified a group of candidate genes involved in cell cycle control and proliferation. For example, the MYC proto-oncogene encodes a transcription factor that plays a critical role in cellular proliferation. Induced rapidly upon entry into the cell cycle, MYC expression is sufficient to drive quiescent cells into proliferation. Additionally, MYC has been shown to be deregulated in a large percentage of human malignancies, and its over-expression frequently correlates with aggressive, poorly differentiated tumors and poor prognosis [207]. Interestingly, enhanced MYC expression was revealed in T_{Reg} cells of RCC patients compared to healthy donors (+2.0) and in T_{Reg} *versus* naive T cells of metastatic RCC (+1.9) in this study, while MYC expression was down-regulated in T_{Reg} compared to naive T cells of healthy donors (-1.7). Paradoxically, in addition to promoting cell proliferation, MYC was shown to sensitize cells to apoptosis under certain conditions [207]. Strikingly, MYC over-expression was thereby inversely correlated with TRAIL sensitivity, which is well in line with the data presented in this work.

CDC25B (cell division cycle 25 homolog B) is a member of the CDC25 family of phosphatases. It activates the cyclin dependent kinase CDC2 (CDK1) by cleaving two phosphate groups and is required for entry into mitosis [208]. CDC25B expression was significantly up-regulated especially in T_{Reg} *versus* naive T cells from metastatic RCC patients (+2.4) and elevated expression levels were also observed when comparing T_{Reg} cells from RCC patients to those from healthy donors (+1.5). CDC25B was not among the significantly differentially expressed candidates discriminating T_{Reg} from naive T cells in healthy donors. Thus, its up-regulation in RCC may contribute to higher $CD4^+CD25^{high}$ T_{Reg} cell frequency in RCC due to enhanced proliferation. Further support for this idea is provided by parallel over-expression of CEB1 (cyclin E binding

protein 1) in T_{Reg} cells from RCC patients compared to healthy donors (+2.1). Increase in CEB1 expression was most significant in T_{Reg} *versus* naive T cells from metastatic RCC patients (+5.2) and CEB1 was not found to be differentially expressed between T_{Reg} and naive T cells of healthy volunteers. CEB1 was demonstrated to interact with various cyclin subunits of CDKs in mammalian cells and its expression was highly elevated by loss-of-function mutations of the tumor suppressor proteins P53 and RB (retinoblastoma). over-expression of CEB1 resulted in up-regulated CDK activity [209], hence facilitating cell proliferation. Another molecule influencing cell proliferation is LST1 (leukocyte-specific transcript 1). LST1 was shown to exert inhibitory effects on lymphocyte proliferation and its expression was increased in affected blood and synovium of rheumatoid arthritis patients [208]. In the presented study, profound down-regulation of LST1 expression was obeserved in T_{Reg} *versus* naive T cells of localized (-1.8) and metastatic (-6.7) RCC patients. As LST1 was described as a negative regulator of lymphocyte proliferation, decreased expression may again indicate elevated proliferation rates of T_{Reg} cells in RCC resulting in increased T_{Reg} cell frequencies. A recent study examining telomere length of $CD4^+CD25^+$ T_{Reg} cells from cancer patients supports this hypothesis [210]. When compared to healthy controls, cancer T_{Reg} cells exhibited significantly decreased levels of TREC (T cell receptor excision circles), but despite their *in vivo* proliferation telomere lengths were not shorter due to induction of telomerase activity. Thus, authors conclude that the increased frequency of T_{Reg} cells in PB of cancer patients is rather caused by active proliferation than by distribution from other compartments (i.e. secondary lymphoid organs or bone marrow). Taken together, these results suggest a combination of decreased susceptibility towards apoptosis and enhanced proliferation to contribute to elevated frequencies of T_{Reg} cells in RCC.

Chemokines are small chemotactic or -attractant cytokines that function as potent activators for leukocyte subpopulations. Their actions are mediated by a family of 7-transmembrane spanning G-protein-coupled receptors growing considerably in size until today. Chemokine receptor (CCR) expression on different cell types as well as their binding and response to specific chemokines are highly variable. The interaction of chemoattractants with leukocytic CCR initiates a series of coordinated biochemical and cellular events including alterations in ion fluxes, integrin avidity and transmembrane potential, changes in cell shape, secretion of lysosomal enzymes, production of superoxide anions, and enhanced locomotion [214]. Tumor secreted chemokines influence tumor metastasis, leukocyte infiltration, regulation of the anti-tumor immune response, angiogenesis and may act as growth factors [215]. Regarding the obtained expression profile of T_{Reg} cells in RCC and health, several chemokine receptors were revealed to be strongly up-regulated. CCR10 expression was elevated in T_{Reg} *versus* naive T cells from healthy donors (+3.2). Its over-expression in T_{Reg} cells increased even more in localized (+5.0) and metastatic (+8.4) RCC depending on disease stage. Furthermore, CCR2, CCR6 and CCR8 were highly up-regulated in T_{Reg} cells compared to their naive counterparts but only in RCC patients. These three CCRs were not among

the significantly differentially expressed genes discriminating T_{Reg} from naive T cells in healthy donors. Induction of all these CCRs may be a hint for an elevated attraction of T_{Reg} cells into the tumor environment that in turn could facilitate tumor escape from immune surveillance. Linehan *et al.* have previously suggested that tumors actively protect themselves from immune destruction by recruitment of T_{Reg} cells [172]. They even proposed tumor-derived factors in its microenvironment that support the chemotaxis, proliferation and/or function of T_{Reg} cells. Coculture of human $CD4^+CD25^+$ T cells with supernatants from primary pancreatic cancer cell lines resulted in significant proliferation of the T_{Reg} cells, while proliferation of $CD4^+CD25^-$, CD4 and CD8 T cells cultured under identical conditions was inhibited. Furthermore, Jonuleit *et al.* showed that soluble factors derived from primary tumor cultures (e.g. IL6, TGF-β, VEGF) significantly impaired DC maturation that in turn led to induction and subsequent increased prevalence of T_{Reg} cells [216]. Tumor-driven up-regulation of T_{Reg} cells in cancer patients could be also caused by the abundance of self-antigens provided by the tumor. It has previously been shown that T_{Reg} cells, which are poorly proliferative *in vitro*, can vigorously proliferate *in vivo* in response to self-antigen stimulation. Furthermore, TGF-β secretion by the tumor was suggested to promote conversion of non-T_{Reg} into T_{Reg} cells [172]. Additionally, tumor-sided expression of chemokines could cause migration of T_{Reg} cells to the tumor microenvironment. In RCC, expression of numerous chemokines was reported to be significantly elevated compared to adjacent normal renal tissue. Among them, one can also find chemokines whose corresponding receptors were revealed to be induced upon T_{Reg} cells from RCC patients [218]. For example, clearly increased CCL4 (chemokine [C-C motif] ligand 4) expression was described in RCC tumor tissue as inversely correlated to tumor size and as a recruiter of lymphocyte infiltration [219]. CCL4, also known as MIP-1B (macrophage-inflammatory protein-1 beta), is a ligand signalling through CCR8, whose expression was significantly induced in RCC T_{Reg} cells compared to their naive counterparts (+3.7; localized:+3.5; metastatic:+4.4) in the presented study. Thus, directed migration of T_{Reg} cells towards the tumor site in RCC patients could be possible. Furthermore, over-expression of the anti-apoptotic chemokine CCL1 that also binds to CCR8 was reported to substantially promote growth of adult T-cell leukemia cells in an autocrine feed back loop [217]. As $CD4^+CD25^+$ human thymocytes also express CCR8, involvement of CCL1-CCR8 interactions in the spread of mature $CD4^+CD25^+$ T cells into circulation was suggested [54]. Interestingly, also MCP-1 (monocyte chemoattractant protein-1, CCL2) gene and protein expression was strongly induced upon CD40 signalling in mouse and human RCC tumor cells showing potent chemotactic activity [220]. This chemokine mediates monocyte chemotaxis and was shown to be involved in monocyte infiltration in the inflammatory response against tumors [208]. CCR2, that is functioning as its receptor, showed elevated expression in RCC T_{Reg} cells (+2.1; +2.2; +2.1) compared to their naive counterparts in this work and could thus also trigger their recruitment to the tumor site. In a mouse model, CCR2 transfected $CD4^+CD25^+Foxp3^+$ T_{Reg}

cells strikingly ameliorated progression of pneumonitis and sialadenitis due to more pronounced migration towards CCL2 expressed in the lung and submandibular gland [221]. As described before, attraction of monocytes and secretion of soluble factors by the tumor could additionally lead to insufficient DC maturation resulting in T_{Reg} cell accumulation [216]. Another chemokine receptor stronger expressed in T_{Reg} cells compaired to naive T cells in RCC patients (+6.2; +5.0; +8.4) than in healthy controls (+3.2) was CCR10, also known as GPR2 (G protein-coupled receptor). CCR10 is predominantly expressed on $CD4^+CLA^+$ (cutaneous lymphocyte antigen) circulating T cells. Its ligand CCL27 is functioning as a homing factor for these memory T cells and is highly expressed in keratinocytes [186]. Thus, CCR10 expression on T_{Reg} cells could be also suggested as a receptor for special homing factors, as the surface phenotype of T_{Reg} cells resembles the one of memory T cells [42]. CCL27 expression was strongly up-regulated in skin lesions of atopic, contact dermatitis, and psoriasis patients. Neutralization of CCL27-CCR10 interactions in mouse models of contact hypersensitivity and atopic dermatitis impaired lymphocyte recruitment and inhibited allergen-induced skin inflammation [186]. Additionally, CCR10 and its ligand CCL27 were demonstrated to contribute to the skin infiltration of malignant T cells in cutaneous T cell lymphoma [222]. CCL28 is another ligand for CCR10, that is highly expressed in human normal and pathological colon, prostate, spleen and to a lesser extent in PBMCs. Interestingly, CCL28 expression could be also detected in mouse kidney. Recombinant human CCL28 displayed chemotactic activity for resting CD4 or CD8 T cells *in vitro* and CCL28-CCR10 interactions were shown to be involved in homeostatic and inflammatory responses in skin and gut [223]. Well in line with the findings presented here, Eksteen *et al.* recently described a subset of CCR10-expressing $CD4^+CD25^+Foxp3^+$ T_{Reg} cells they isolated from chronically inflamed human liver. Interestingly, these cells were detected around bile ducts that expressed increased CCL28 levels due to epithelial inflammation and migration towards this chemokine was confirmed *in vitro*. Authors conclude that CCR10 allows T_{Reg} cells to respond to CCL28 secreted by epithelial cells resulting in the accumulation of $CCR10^+$ T_{Reg} cells at mucosal surfaces [224]. Similarly, attraction of T_{Reg} cells could be caused by potential secretion of CCL28 by the tumor in RCC patients. Furthermore, expression of CCR6 and its ligand CCL20 were strongly up-regulated in hepatic, colorectal and pancreatic cancer and were described to contribute to carcinogenesis and progression of these malignancies [225]. Elevated expression of CCR6 was also observed in $CD4^+CD25^+$ T_{Reg} cells compared to $CD4^+CD25^-$ naive T cells in RCC (+4.0; +3.5; +4.9) but not in healthy donors in this study. In accordance with these results, Hirahara *et al.* recently reported that the majority of human PB $CD4^+CD25^+Foxp3^+$ T_{Reg} cells bear functional skin homing receptors such as CCR4, CCR6, CCR10 and demonstrated chemotactic responses of these cells towards their particular ligands [226]. Apart from mediating homing to skin, it would be also conceivable, that expression of these receptors is induced during carcinogenesis in RCC to recruit T_{Reg} cells to the tumor and to facilitate

its evasion from immune surveillance. That appropriate localization is indispensably required for *in vivo* T_{Reg} cell function and that migration behaviour of T_{Reg} cell subsets influences their *in vivo* suppressive capacity was recently described by Huehn and co-workers [227]. Thus, aberrant homing of RCC T_{Reg} cells may also cause enhancement of their regulatory abilities in the tumor environment and together with elevated T_{Reg} cell frequencies tumor immune escape could be supported. Further prove for this hypothesis is obtained by the observed induction of integrin expression in T_{Reg} from RCC patients. ITGB1 (integrin beta 1; +2.1; +2.0; +2.4) and ITGB5 (+3.1; +2.7; +2.8) expression were significantly up-regulated in RCC T_{Reg} compared to naive T cells, but failed to show differential expression between both T cell subsets in healthy donors. The integrins comprise a large family of cell-surface proteins that mediate adhesion between cells, and between cells and the extracellular matrix. Interestingly, T cell integrins are activated by chemokine signalling to bind tightly to vascular surfaces during the migration of activated lymphocytes into sites of inflammation [29]. Elevated expression of ITGB5 has recently been described also in PB monocytes that migrate to and accumulate in hypoxic areas of inflammatory and tumor lesions [228]. The ICAMs (intracellular adhesion molecules) are members of the immunoglobulin superfamily and serve as cell surface ligands for the leukocyte integrins [29]. ICAM1 is involved in enhancement of tumor metastasis and mediates lymphocytic tumor cytotoxicity. Interestingly, ICAM1 expression was shown to be up-regulated in response to cytokines and through CD40 signalling in RCC [230, 229]. Another important adhesion molecule, known to bind integrins on T cells is VCAM1 (vascular cell adhesion molecule 1). It is a cell surface glycoprotein implicated in various pathophysiologic conditions and VCAM1 expression was reported to be significantly elevated in clear cell and papillary RCC compared to adjacent normal renal tissue [232]. In breast cancer patients, up-regulation of VCAM1 expression in tumor cells additionally correlated with short survival time [231]. A very recent report interestingly demonstrated that retroviral transfer of VCAM1 into an immune-susceptible cancer cell line significantly increased its resistance towards vaccine-induced immune response. In parallel, the number of tumor-infiltrating $CD8^+$ T cells was dramatically decreased in tumors expressing VCAM1, although *in vitro* transwell migration assays showed the ability of VCAM1 to promote $CD8^+$ T cell migration. While most RCCs analysed expressed VCAM1, an RCC responding to vaccination did not. Thus, authors conclude, that tumor expression of VCAM1 represents a new mechanism of immune evasion and may have important implications for the development of immunotherapy for human RCC treatment [233]. Linking these findings to the results obtained in the presented study, up-regulation of CCRs and integrins on RCC T_{Reg} cells accompanied by tumor-sided expression of their specific ligands could indeed lead to tumor-driven attraction of T_{Reg} cells and their accumulation at tumor-sites. The particular chemokines or adhesion molecules expressed by the tumor may simultaneously impair migration of tumor antigen-specific cytotoxic lymphocytes into malignant RCC tissue and attracted T_{Reg} cells could prevent tumor rejection as actually intended.

In addition to decreased apoptotic sensitivity, increased proliferation and migration to tumor sites, an augmented suppressive activity of T_{Reg} cells may be supposed to contribute to tumor immune escape. Studies examining inhibitory capacities of T_{Reg} cells in cancer patients gave contradictory results. While some authors claim similar regulatory function of T_{Reg} cells from cancer patients and healthy controls [198, 40], other reports could indeed demonstrate higher immunosuppressive competence of T_{Reg} cells in cancer patients. T_{Reg} cells infiltrating metastatic lymph nodes in melanoma patients were shown to suppress proliferation of $CD8^+$ and $CD4^+CD25^-$ T cells more potently than T_{Reg} cells sorted from tumor-free lymph nodes of the same patient. Furthermore, these T_{Reg} cells strongly inhibited IL2 and IFN-γ production of naive T cells [234]. Compared to healthy donors, T_{Reg} cells from patients suffering from epithelial malignancies have additionally been reported to exert increased inhibition of $CD4^+$ T cell proliferation [85].

4.3.3 More news about the phenotype of human T_{Reg} cells in RCC

Using quantitative RT-PCR, confirmation of the microarray approach could be extended to the analysis of human regulatory and naive T cells isolated from RCC patients. Comparable to results obtained in healthy donors, significantly elevated mRNA expression of FOXP3, CCR10, TNFRSF1B, TRAF1 and LGALS3 was verified in T_{Reg} *versus* naive T cells of RCC patients. Well in line with these results, increased FOXP3 mRNA levels were reported previously in T_{Reg} compared to naive T cells isolated from cancer patients suffering e.g. from CLL [41], metastatic melanoma and even RCC [198]. No statistically significant differences in FOXP3 mRNA levels were observed between healthy volunteers and RCC patients due to high variability among donors, although mean fold changes in RCC patients (+19.2) were somehow higher compared to healthy controls (+11.6). Reports on that issue are also contradictory. For example, Dannull *et al.* claimed significantly higher FOXP3 mRNA copy numbers in T_{Reg} cells from RCC patients [95], whereas Cesana *et al.* found no differences in FOXP3 mRNA expression between RCC patients and healthy controls [198]. To assess correlation of mRNA and protein expression levels, PBMCs from RCC patients were three-colour stained and subjected to FACS analyses. Thereby, a significant increase in FOXP3 and LGALS3 protein expression in RCC patient derived T_{Reg} compared to naive T cells could be confirmed. In agreement with these findings, elevated FOXP3 protein expression in RCC T_{Reg} *versus* naive T cells has been reported [198, 95]. In ovarian cancer tissue samples, FOXP3 mRNA expression was even suggested as a surrogate marker for indirect quantitation of the T_{Reg} cell infiltration grade into tumor tissue. Thereby, high expression levels of FOXP3 have been associated with a dismal prognosis as well as with an inferior overall and progression-free survival [91, 210]. Consistent with other reports, comparison of FOXP3 protein amounts between healthy control and RCC patient derived T_{Reg} cells revealed similar expression levels in both donor groups [198, 95]. Both RCC studies analysed expression of additional T_{Reg} cell-associated surface molecules, such as GITR and CTLA4, that were found to be up-regulated on patient T_{Reg} *versus*

naive T cells, but showed similar expression levels when compared to healthy donors. Furthermore, this work is the first publication reporting elevated LGALS3 protein expression in RCC T_{Reg} cells as well as increased fractions of $CD4^+$ T cells coexpressing FOXP3 and LGALS3 or FOXP3 and TNFRSF1B when compared to healthy donors. In healthy donors, clear up-regulation of LGALS3 protein expression has previously been demonstrated upon activation in T_{Reg} cells [197]. Antigenic stimulation mediated by a tumor, could be similarly supposed to activate T_{Reg} cells in RCC patients that in turn react with a significant up-regulation of LGALS3 expression.

As discussed before [chapter 4.2.3], LGALS3 and TNFRSF1B have been selected as potential new marker genes enabling discrimination of human regulatory from naive T cells. As both genes were highly up-regulated in RCC as well as in healthy donor derived T_{Reg} *versus* naive T cells, their qualification as new T_{Reg} cell markers is further underlined. These genes belong to the ones differing most between T cell subpopulations, even independent from their origin. In this context, CCR10 again attracts attention as it was found to be clearly over-expressed on RCC T_{Reg} cells compared to healthy donors and as it could function as a very convenient marker molecule due to its expression on the cell surface. As exact enumeration of T_{Reg} cells in RCC is complicated by the fact that $CD4^+$ T cells with negative or intermediate CD25 expression levels may up-regulate CD25 on their surface in response to antigenic stimulation by the tumor [95], these molecules could perhaps facilitate detection of T_{Reg} cells. Overcoming the lack of specific cell surface markers could help to clarify many functionally relevant aspects of T_{Reg} cells in tumor immunology that are currently still unknown. Although research in T_{Reg} cell biology is intensifying, it is e.g. still not clear, whether T_{Reg} cells in human tumors are primed mainly in the thymus or emerge in the periphery due to antigen-specific stimulation [63].

Similar to the presented findings in healthy donors, reduced susceptibility of RCC T_{Reg} cells towards apoptosis could be confirmed when compared to their naive counterparts. Unexpectedly, no significant differences in apoptotic behaviour was observed when comparing RCC to healthy donor derived T_{Reg} cells. Thus, elevated frequencies of T_{Reg} cells in RCC patients seem to be caused rather by an increase in proliferation than by a decrease in incidence of apoptotic cell death (for detailed discussion see chapter 4.3.2). In AML and SCCHN (squamous cell carcinoma of head and neck) patients, PB-derived T_{Reg} cells even contained significantly more apoptotic cells when compared to healthy donors but increased proliferation rates of T_{Reg} cells indeed compensated for this loss resulting in elevated T_{Reg} cell frequencies [104]. This T_{Reg} cell expansion was suspected to be tumor-driven, e.g. as a response to mostly self-antigens derived from the tumor cells [172]. Paradoxically, naive T cells in the RCC patients analysed in this study clearly showed higher fractions of apoptotic cells than naive T cells in healthy donors. As discussed in detail in the previous chapter, this fact may point to increased induction of apoptosis in effector T cells mediated by T_{Reg} cells or the tumor itself that could in this way facilitate tumor immune escape.

4.4 Regulatory T cells in IBD

The gastrointestinal tract is the main interface where the body encounters exogenous antigens. It is crucial and challenging that local responses are tightly regulated to avoid an immune reaction against dietary antigens and commensal flora while still mounting an efficient response against pathogens. Faults in maintenance of intestinal homeostasis may lead to disease, inducing local and often also systemic inflammation [74]. The complex pathophysiologic mechanisms that trigger and maintain the two forms of IBD, CD and UC, remain only partially understood. However, a large body of data from animal models and functional studies in humans indicate that IBD is caused by an imbalance of the immune system characterized by an excess of inflammatory stimuli and mediators accompanied by an inadequate low number or function of cellular components that down-regulate mucosal immune responsiveness. As regulatory T cells control responses to self- and foreign antigens, they were demonstrated to be involved in oral tolerance, to inhibit the activation of T_H1 cells against enteric bacterial antigens and to prevent and treat established colitis in animal models of IBD [235]. Considering the potent regulatory capacity of $CD4^+CD25^{high}$ peripheral blood T cells in healthy human individuals and the potential of microarrays to decipher gene regulation events that might contribute to IBD pathogenesis, T_{Reg} *versus* naive T cells of IBD patients were compared applying the *Human T_{Reg} Chip*. To our knowledge, this is the first extensive analysis of gene expression in human T_{Reg} cells from IBD patients, although different microarray studies investigating IBD have been previously performed. Intestinal mucosa samples have been subjected to several exploratory gene expression analyses aiming at improving classification between CD and UC as well as unravelling gene regulation events leading to IBD pathogenesis [9]. Burczynski *et al.* applied microarray analyses to PBMCs from IBD patients as these cells represent the more clinically accessible tissue and identified a set of 12 genes reliably discriminating CD from UC patients [239]. Similar to findings obtained during the presented study, the number of regulated genes was clearly higher between CD patients and healthy donors compared to UC patients *versus* healthy controls.

Transfer of T_{Reg} cells cannot only prevent the development of colitis in animal models but can even cure established disease by acting on adaptive as well as innate immune system. It has been demonstrated that T_{Reg} cells suppress T cell activation and proliferation in the lymphoid organs, thereby avoiding establishment of the disease. Furthermore, they are able to accumulate in the inflamed colon, curtailing the mucosal inflammatory response while also reducing immune activation in the lymphoid organs in order to counteract disease. These processes do not necessarily employ the same effector mechanisms, and they clearly have different migratory requirements. In prevention of colitis, T_{Reg} cells have to control activation of a predominantly naive population of cells, while in cure of colitis, they have to deal with antigen-experienced cells and an aggressive inflammatory response. Although the knowledge about the migratory behaviour of immune cells has greatly increased in recent years, we are just

starting to unravel what drives which cells in which direction. The tissue specificity of activated and memory T cells is reversible, and has been demonstrated to be imprinted in secondary lymphoid organs. T cells activated in mesenteric lymph nodes (MLNs) or Peyer's patches exclusively will display gut tropism and DCs isolated from these secondary lymphoid organs are sufficient to induce intestinal homing in T cells. However, the mechanism by which DCs mediate tissue imprinting is still poorly understood. A significant breakthrough has been the discovery that retinoic acid production by DCs is essential for the induction of gut homing receptors on T cells [74]. Strikingly, an exclusive and significant down-regulation of RXRA (retinoid X receptor alpha) was observed in IBD-derived *versus* healthy T_{Reg} cells (-4.2). RXRA is a nuclear hormone receptor that mediates biological effects of retinoids by its involvement in retinoic acid-induced gene activation. Thus, decreased expression of RXRA in IBD T_{Reg} cells could lead to less responsiveness towards retinoic acid signalling and in consequence to impaired gut-homing of T_{Reg} cells. This hypothesis is supported by recent results of Maul and co-workers reporting an insufficient increase of $CD4^+CD25^+$ T_{Reg} cells in intestinal lesions from IBD patients compared to diverticulitis patients to efficiently counteract disease [235]. The two main gut-associated homing molecules described to date are the chemokine receptor CCR9 and integrin $\alpha_4\beta_7$, that in concerted action with expression of their ligands CCL25 and Mad-CAM1 help directing lymphocytes into the intestine. Unfortunately, both molecules were not included in the oligonucleotide pool constituting the applied *Human T_{Reg} Chip* thus, potential involvement of altered CCR9 and/or $\alpha_4\beta_7$ expression in impaired homing of T_{Reg} cells towards the intestine remained in the dark. But although CCR9 and $\alpha_4\beta_7$ are important for intestinal lymphocyte migration, they are neither exclusive for that tissue nor absolutely essential for gut localization [74]. Following activation in MLNs, primed murine T cells were demonstrated to acquire a chemokine receptor profile including CCR9, CCR6 and CXCR3 enabling them for homing to the small intestine. However, $CCR9^{-/-}$ T cells also showed migration to the lamina propria probably due to compensation by paralleled expression of CCR2, CCR5, CCR6, CCR8, CCR10, CXCR3 and CXCR6 [236]. When comparing T_{Reg} *versus* naive T cells in healthy donors and IBD patients, different chemokine receptor expression patterns were strikingly observed. While CCR5 (+2.2) and CCR10 (+3.3) were up-regulated, CCR7 (-2.1) was revealed to be significantly down-regulated in a comparison of both T cell subsets in healthy donors. In contrast to that, a significant over-expression of CCR6 (+2.0), CCR8 (+3.6) and an even higher up-regulation of CCR10 (+5.6) was disclosed in IBD-derived T_{Reg} *versus* naive T cells. Furthermore, CCR4 expression (-6.3) was clearly decreased in a direct comparison of IBD and healthy T_{Reg} cells. Considering these tremendous differences in chemokine receptor expression signatures between healthy volunteers and IBD patients and the fact that different chemokine receptors mediate distinct migratory properties of T cell subsets it is conceivable that IBD T_{Reg} cells may show aberrant migration behaviour. In this context, it is worth noticing that a strong down-regulation in expression of various chemokines

was also observed, such as CCL3 (MIP1α, -6.4 in CD inactive, -8.5 in UC inactive), CCL4 (-10.8 in active CD, -4.5 in inactive CD) and CCL5 (RANTES, -5.6 in IBD, -2.5 in health) in T_{Reg} *versus* naive T cells from IBD patients. However, several reports claim accumulation of FOXP3$^+$ T_{Reg} cells in inflamed tissues in IBD patients that is suggested to be driven by inflammation in the intestine which would be well in line with the demonstrated observation of elevated expression of CCR6, CCR8 and CCR10 in T_{Reg} cells from IBD patients. Authors argue that the presence of FOXP3$^+$ T_{Reg} cells underlines possible impotent regulatory capacity or nonresponsiveness to their activity under conditions of chronic intestinal inflammation [238, 235]. Thereby, clear evidence that a lack of functionally sufficient T_{Reg} cells can lead to chronic intestinal inflammation emerged from a study of IPEX patients [238].

Maul *et al.* reported increased presence of T_{Reg} cells in PB of IBD patients in remission compared with active disease and decreased T_{Reg} cell frequencies in active IBD *versus* diverticulitis patients [235]. As these discrepancies in T_{Reg} cell numbers may be attributed to differences in apoptosis and/or proliferation rates, having a closer look on the presented gene expression patterns comparing T_{Reg} *versus* naive T cells of active and inactive CD patients as well as inactive UC patients was especially interesting. Surprisingly, TNFRSF25 expression was significantly elevated (+1.9) in T_{Reg} cells *versus* naive T cells from active CD patients, but was not differentially expressed between both T cell subsets in the remaining patient groups and healthy volunteers. TNFRSF25 is also known as death receptor 3 (DR3) and was shown to regulate cell apoptosis and thus lymphocyte homeostasis *via* stimulation of NF-kappa B activity. TNFRSF25 knockout studies in mice interestingly suggested an involvement of this gene in the removal of self-reactive T cells in the thymus and gene duplication was demonstrated to be associated with rheumatoid arthritis. Upon T cell activation, alternative splicing of TNFRSF25 results in synthesis of then predominantly membrane-bound isoforms controlling lymphocyte proliferation [208]. In contrast to that, TNFRSF1B exerts anti-apoptotic activity and was revealed to be over-expressed in T_{Reg} *versus* naive T cells of IBD patients in remission (+2.5 for inactive CD, +2.1 for inactive UC) but not in active IBD, thereby reaching similar fold changes like in healthy donors (+2.5) and RCC patients (+2.1). Strikingly, two polymorphisms in the TNFRSF1B gene were described to contribute greatly to an increased risk of CD, even correlating with disease severity and responsiveness to therapy [82]. Comparison of T_{Reg} *versus* naive T cells for all IBD patients independent from disease stage revealed exclusive over-expression of another pro-apoptotic molecule, namely TNFRSF6 (Fas receptor, +2.2). Interestingly, increased expression of its ligand TNFSF6 has been reported in IBD colonic lamina propria thus maybe delivering required apoptotic stimuli [244]. Furthermore, platelet endothelial cell adhesion molecule-1 (PECAM1) is a homophilic-binding member of the immunoreceptor tyrosine-based inhibitory motif (ITIM) family of inhibitory receptors that functions prominently to prevent apoptosis in naturally occurring vascular subjected to programmed cell death stimuli. Human T lymphocytes lacking PECAM1

were shown to be far more sensitive than their PECAM1 expressing counterparts to multiple death signals, suggesting PECAM1 as a novel and potent suppressor of BAX mediated apoptosis [240]. Strikingly, PECAM1 belonged to the genes showing the strongest decrease in expression for a comparison in T_{Reg} and naive T cells from IBD patients (-5.2), thereby demonstrating a far more pronounced down-regulation in active CD (-7.4) than in inactive UC (-3.3). In contrast to that, PECAM1 expression was only very slightly diminished between T_{Reg} and naive T cells (-1.4) in healthy donors. Taken together, these findings could at least in part explain different T_{Reg} cell frequencies in IBD patients depending on disease state.

Another defective mechanism in CD is T cell resistance to apoptosis, leading to inappropriate immune homeostasis and accumulation of activated T cells in the tissues enhancing inflammation. T_{Reg} cell induced apoptosis of effector T cells has recently been suggested as a potential mechanism of T_{Reg} cell mediated immunosuppression and was demonstrated to be dependent on GZMA, perforin and ITGB2. Interestingly, a significant down-regulation of GZMA was observed in T_{Reg} *versus* naive T cells in IBD patients suffering from active (-3.6) and inactive (-3.7) CD, while ITGB2 expression was slightly decreased in UC patients in remission (-1.5). In this context, the galectins LGALS1 and LGALS3 may also play a role. LGALS1 was demonstrated to be able to induce apoptosis in activated T cells and was only found to be significantly differentially expressed between T_{Reg} and naive T cells from UC patients in remission, but not in patients with active IBD. Similarly, LGALS3 expression was not revealed to be significantly altered when comparing T_{Reg} *versus* naive T cells in all IBD patient cohorts examined, whereas its expression has been disclosed to be clearly up-regulated between both T cell subsets in healthy donors (+3.9). As described previously, LGALS3 may act as a pro-apoptotic molecule when secreted extracellularily. Furthermore, CLL5 was the most down-regulated gene in T_{Reg} *versus* naive T cells isolated from IBD patients (-5.6) showing a far less decrease in expression between both T cell subsets in healthy donors (-2.5). Micromolar concentrations of CCL5 have been demonstrated to induce apoptosis in human PB-derived T cells *via* the CCR5 chemokine receptor, that is interestingly up-regulated upon T cell activation [243]. Taken these results together, it is conceivable that impaired T_{Reg} cell mediated apoptosis induction in T effector cells may contribute to IBD pathogenesis due to persistence of activated intestinal T cells.

Another interesting molecule revealed by the extensive microarray study of human T_{Reg} and naive T cells in IBD is interleukin-23. IL23 is a heterodimeric cytokine encoded by IL23A and the p40 subunit of IL12 (IL12B). IL23 can activate STAT4 expression and stimulate the production of IFN-γ. While IL12 is mainly acting on naive $CD4^+$ T cells, IL23 predominantly exerts its function on memory $CD4^+$ T cells [208]. Consistent with previous studies reporting elevated IL23 expression in CD mucosa, exclusive up-regulation of IL23A expression was found in T_{Reg} *versus* naive T cells from CD patients with active disease. Whereas the central role of IL12 in the generation

of IFN-γ secreting cells and thus in the regulation of T_H1 responses has long been appreciated, there is now emerging evidence for an even more important role of IL23 in the manifestation of intestinal inflammation. Mouse study results with targeted deletion of the IL23/p19 subunit suggested that IL23-driven intestinal inflammation is mediated by production of IL6 and IL17. Moreover, administration of recombinant IL23 accelerated the development of colitis in lymphocyte-deficient RAG knockout mice after reconstitution with $CD4^+$ T cells from $IL10^{-/-}$ mice [237]. Elevated expression of IL23A has additionally been reported in psoriatic lesions compared with nonlesional skin and IL23 has been implicated in recruitment of inflammatory cells in T_H1 mediated autoimmune and chronic inflammatory diseases [208]. In summary, these results suggest a contribution of T_{Reg} cell secreted IL23A to a shifted balance towards T_H1 responses as seen in CD.

CD and UC have both been associated with disturbances in vascular physiology, including permeability and angiogenesis, that are in part regulated by endothelial intercellular junctions. These junctions are composed of several adhesion molecules, including PECAM1. Lower levels of this versatile cell adhesion molecule in its soluble form were described in intestinal biopsies from patients suffering from active UC with extensive location [241]. Furthermore, PECAM1 plays a significant role in leukocyte transendothelial migration by mediating homophilic interactions between endothelial cells (EC) as well as between ECs and leukocytes, thus preserving vessel integrity. Increased PECAM1 expression has been demonstrated in patients suffering from active multiple sclerosis, neuroinflammation due to HIV infection and PECAM1 was shown to be involved in Alzheimer disease pathogenesis [242]. As previously described, decreased PECAM1 levels were revealed in T_{Reg} *versus* naive T cells from IBD patients (-5.2) that were additionally far less pronounced in healthy donors (-1.4) thereby perhaps indicating alterations in EC-leukocyte interactions contributing to IBD pathogenesis.

5 Summary and Perspectives

Regulatory T cells have a far-reaching effect on our health by influencing the outcome of infection, autoimmunity, graft survival, cancer, and even allergy. Despite recent advances in T_{Reg} cell immunology, requirements for their development, maintenance, and mode of action in humans remain poorly defined as thus far, genomic studies on T_{Reg} cells were restricted to murine models. To improve molecular characterization of human T_{Reg} cells, a unique microarray has been compiled, the *Human T_{Reg} Chip*, consisting of 350 T_{Reg} cell associated genes. To ensure measurement of accurate and reliable transcription profiles, this chip was validated in terms of cross-platform comparison to Affymetrix, reproducibility in repeated experiments, reliability expressed as CV, spotting quality, linearity of hybridization, and sensitivity. During this evaluation process the *Human T_{Reg} Chip* emerged as a reliable and suitable tool to the scientific community facilitating research on human T_{Reg} cells in health and disease. Afterwards, purity and regulatory phenotype, expressed as fraction of FOXP3 positive cells and suppressive capacity, of MACS separated human $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ naive T cells was confirmed. Microarray analyses were performed by separately hybridizing T_{Reg} or naive T cell-derived cRNA of 11 healthy volunteers, yielding 62 differentially expressed genes after application of normalization and SAM algorithms to the data set. Among them, expression of 21 "old friends" previously described in murine and/or human T_{Reg} cells lent greater creditability to the identification of 41 "new players" that have not been mentioned in the characterization of T_{Reg} cells at all before. Application of PathwayAssist software to the unique data set, revealed implication of these candidates in functional key modules pointing to mechanisms controlling diverse cellular processes such as survival/apoptosis, TCR signalling/activation/proliferation and differentiation/maintenance of human T_{Reg} cells in health. Furthermore, the vast majority of genes have been reported to be involved in autoimmunity, tumor development, transplantation tolerance and even allergy - all representing disease scenarios thought to result at least partly from unbalanced T_{Reg} cell activity. T_{Reg} cell specific expression of five selected genes-of-interest, namely LGALS3, CCR10, TNFRSF1B, CCR5 and TRAF1 was confirmed by real-time RT-PCR. Furthermore, FACS analysis confirmed correlation of TNFRSF1B, CCR10, LGALS3 and FOXP3 gene and protein expression and demonstrated significantly higher amounts of TNFRSF1B, LGALS3 and FOXP3 proteins in human $CD4^+CD25^+$ regulatory compared to $CD4^+CD25^-$ naive T cells in healthy donors. LGALS3 as well as TNFRSF1B proteins were revealed to be coexpressed with FOXP3 in a considerable fraction of $CD4^+$ T cells, although an even more extended population just coexpressed CD4 and one of the both proteins-of-interest. It would be interesting to figure out, whether and how these T cell subsets differ in their regulatory phenotype. Therefore, $FOXP3^+LGALS3^{high/low}$ and $FOXP3^+TNFRSF1B^{high/low}$ $CD4^+$ T cells should be sorted and compared in mixed lymphocyte reactions regarding their suppressive capacity. During advanced analyses

of the five selected genes/proteins-of-interest, LGALS3 and TNFRSF1B emerged as two new putative T_{Reg} cell marker molecules, as their mRNA as well as protein over-expression in healthy human T_{Reg} cells was sustained at constantly high levels even after *in vitro* activation with anti-CD3. Moreover, expression of both genes/proteins were not considerably induced upon activation in $CD4^+CD25^-$ naive T cells and remained clearly lower than in activated T_{Reg} cells. Thereby, expression behaviour of LGALS3 and TNFRSF1B resembled the one of FOXP3 supporting the role of both molecules as new human T_{Reg} cell markers. Next, susceptibility to apoptotic cell death was compared in T_{Reg} *versus* naive T cells from healthy donors as microarray analyses revealed several genes participating in apoptosis control. Applying combined Annexin V and propidium iodide staining, a lower apoptotic sensitivity of the regulatory T cell fraction could indeed be demonstrated when compared to their naive counterparts in healthy volunteers. As admittedly, the number of measured FACS events were rather low due to limited cell numbers and the obtained data was probably highly influenced by the applied gating strategy, results should be reassured by performing additional apoptosis assays.

Using the expression signature of human T_{Reg} cells from healthy donors as a reference, it was interesting to analyse commonalities and differences in T_{Reg} cell biology in cancer and chronic inflammatory disease. First, T_{Reg} cells from RCC patients were investigated, suffering either from localized or metastatic tumor growth. Significant higher $CD4^+CD25^{high}$ T_{Reg} cell frequencies could be demonstrated in RCC patients compared to healthy donors, but no substantial differences in T_{Reg} cell numbers were found between localized and metastatic RCC. Thereby, elevated T_{Reg} cell prevalence in RCC was accompagnied by an increase of total $CD4^+CD25^+$ T cells, incorporating $CD25^{low}$ as well as $CD25^{dim}$ T cells. Suppressive capacity of T_{Reg} cells should be compared between RCC patients and healthy donors to evaluate whether T_{Reg} cells in RCC display functional deficits in addition to their numerical excess. Gene expression analyses of RCC T_{Reg} and naive T cells applying the *Human T_{Reg} Chip* were performed and allowed comparison of transcriptional patterns between healthy donors and RCC patients as well as comparison of expression signatures between localized and metastatic tumor growth. Although no genes with opposed regulations could be revealed between all patient cohorts, numerous genes were detected to be exclusively differentially expressed in T_{Reg} *versus* naive T cells in only one of the donor groups. Comparing T_{Reg} *versus* naive T cells, the vast majority of genes interestingly showed stronger regulation in RCC patients than in healthy donors and in metastatic than in localized tumor growth. Furthermore, a rising number of regulated genes was identified comparing both T cell subsets in healthy donors *via* localized to metastatic RCC patients. As especially the fraction of over-expressed candidates increased dramatically depending on stage of disease, a gain-of-function in RCC patient-derived T_{Reg} cells may be concluded. Dissecting the gene expression pattern of RCC T_{Reg} and naive T cells disclosed several mechanisms probably supporting tumor immune escape. Numerous

genes were identified contributing to enhanced survival of RCC T_{Reg} cells due to decreased susceptibility towards apoptosis. Together with candidates enabling increased proliferation rates of T_{Reg} cells in RCC, these findings may provide a possible explanation for elevated T_{Reg} cell frequencies in RCC. Furthermore, exclusive induction of several homing receptors and adhesion molecules was observed in RCC-derived T_{Reg} cells that may lead to elevated attraction of T_{Reg} cells into the tumor environment. To prove this hypothesis, it would be interesting to evaluate chemokine expression in RCC tumors as these small chemotactic cytokines mediate T_{Reg} cell migration. Furthermore, migration assays and immunohistochemistry comparing expression of the induced chemokine receptors on tumor infiltrating T_{Reg} cells and on T_{Reg} cells in adjacent healthy renal tissue could add exiting news about the homing behaviour of RCC T_{Reg} cells. Moreover, several genes were revealed to be able to confer exaggerated suppressive capacity to T_{Reg} cells in RCC, such as an augmented ability to induce apoptotic cell death in anti-tumor effector T cells. Taken together, all these functional modules could act in concert to promote T_{Reg} cell mediated tumor immune evasion. Correlation of microarray and quantitative real-time RT-PCR results was demonstrated for FOXP3 as a well-characterized T_{Reg} cell gene and for the selected "new players" LGALS3, CCR10, TNFRSF1B and TRAF1. Comparing T_{Reg} *versus* naive T cells, a significant decrease in the fold change of LGALS3 expression was observed in RCC patients. Similar to healthy donors, FACS analyses revealed clearly elevated FOXP3 and LGALS3 protein expression levels in T_{Reg} *versus* naive T cells from RCC patients. While the fraction of FOXP3 positive cells within the $CD4^+CD25^{high}$ T_{Reg} subpopulation was comparable between healthy donors and RCC patients, a significantly higher percentage of LGALS3 expressing cells was strikingly discovered among the RCC T_{Reg} cells. This result could point to an antigenic stimulation of RCC T_{Reg} cells mediated by the tumor, as LGALS3 expression was demonstrated to be clearly induced upon activation of T_{Reg} cells in healthy donors [197]. Well in line with this finding, clearly larger $CD4^+$ T cell subpopulations were detected coexpressing LGALS3 and FOXP3 as well as TNFRSF1B and FOXP3 in RCC patients compared to healthy volunteers. As observed for healthy donors, staining for Annexin V and propidium iodide with subsequent FACS analyses revealed that RCC T_{Reg} cells were also less susceptible to apoptosis than their naive counterparts. In contrast to that, an enhanced protection from programmed cell death could not be confirmed in RCC *versus* healthy donor-derived T_{Reg} cells, thus elevated frequencies of T_{Reg} cells in RCC seem to be rather caused by an increase in proliferation. To prove this, proliferation rates in RCC- *versus* healthy donor-derived T_{Reg} cells should be assessed in appropriate proliferation assays. Paradoxically, higher apoptotic rates were detected in naive T cells from RCC patients, supporting the presented idea of an increased T_{Reg} cell-mediated apoptosis induction in effector T cells contributing to tumor immune escape. As the composition of cells in the blood stream might not be comparable to the conditions at the site of tumor development, comparison of gene expression patterns from PB-derived to tumor infiltrating

T_{Reg} cells in RCC patients could be very interesting. Furthermore, T_{Reg} cell numbers and changes in gene expression from RCC patients should be investigated before and after cytoreductive nephrectomy, as the primary tumor (not only) in RCC has been recognized to suppress anti-tumor effects [199, 40].

Since T_{Reg} cells have proven effective in the prevention and down-regulation of IBD in animal models, abnormalities in their suppressor function, frequency or distribution in the intestinal mucosa are supposed to contribute to IBD pathogenesis [235]. These facts led us to have a closer look on T_{Reg} cells isolated from IBD patients, suffering either from UC or CD and presenting active or inactive state of disease. In cooperation with Dr. Maul, decreased PB T_{Reg} cell frequencies were demonstrated in active IBD, whereas T_{Reg} cells were enriched in PB of IBD patients presenting with inactive state of disease. Although T_{Reg} cell numbers were increased in inflamed intestinal lesions *versus* noninflamed areas, T_{Reg} cell presence was only insufficiently enhanced compared to diverticulitis patients [235]. To decipher gene regulation events and to identify novel genes that might be involved in IBD pathogenesis, maintenance and progression, comparative microarray analyses of $CD4^+CD25^+$ T_{Reg} and $CD4^+CD25^-$ naive T cells were performed isolated from PB of IBD patients suffering from active CD, inactive CD or inactive UC. Similar to results obtained from RCC patients, comparing this IBD T_{Reg} cell fingerprint to the obtained healthy reference expression pattern did not reveal genes with opposite directions in regulation but several candidates exclusively differentially expressed between T_{Reg} and naive T cells in only one donor group. Genes that were found to be regulated in both donor cohorts, again showed generally stronger alterations in IBD patients. Direct comparison of T_{Reg} cells between IBD patients and healthy donors disclosed far more down-regulated than up-regulated genes that could point to loss-of-functions in IBD-derived T_{Reg} cells contributing to insufficient regulatory capacity and thus to IBD pathogenesis. This extensive IBD microarray study even allowed comparative analysis of T_{Reg} cells from IBD patients suffering from different stage of disease, such as active/inactive CD and inactive UC. Interestingly, the number of genes differentially expressed between T_{Reg} and naive T cells significantly declined from active to inactive disease state, supporting the idea of a rapprochement in T_{Reg} cell gene expression of IBD patients in remission to healthy values. Strikingly, tremendous differences in chemokine receptor expression patterns were found between T_{Reg} and naive T cells in health and IBD that could result in impaired homing of T_{Reg} cells towards the intestine. Investigation of IBD- and healthy donor-derived T_{Reg} cells in migration assays could thereby gain new insights into migration behaviour of T_{Reg} cells in chronic inflammatory diseases. Furthermore, revelation of significant over-expression of several genes with pro-apoptotic functions and down-regulation of anti-apoptotic genes in active IBD compared to patients in remission or healthy donors could lead to insufficient T_{Reg} cell numbers as observed in IBD. Moreover, a functional module of differentially expressed genes was detected suggesting impaired T_{Reg} cell mediated apoptosis induction in T effector cells thus leading to accumulation of activated T cells

in gut tissues and enhancing inflammation in IBD. To prove this hypothesis, T_{Reg} cells from IBD patients and healthy volunteers should be compared regarding their apoptotic susceptibility and their ability to induce programmed cell death in T effector cells in appropriate apoptosis assays. Another interesting module disclosed during dissection of the gene expression patterns characterizing T_{Reg} cells in IBD indicated alterations in EC-leukocyte interactions. As conditions in the blood stream obviously differ from the ones at the site of inflammation, gene expression of gut-infiltrating lymphocytes should be analysed as well and should be compared to the transcriptional pattern obtained from PB T_{Reg} cells. Again, quantitative real-time RT-PCR confirmed the microarray data for FOXP3, LGALS3, CCR10, TNFRSF1B and TRAF1 gene expression. Thereby, a significantly decreased fold change in expression of LGALS3 and TNFRSF1B was observed in IBD-derived T_{Reg} *versus* naive T cells when compared to healthy donors, whereas FOXP3 showed a clearly elevated fold change in the same comparison.

Taken together, the comprehensive sets of genes discriminating human regulatory from naive T cells provide a defined starting point to unravel the unique characteristics of T_{Reg} cells in health and disease. The *Human T_{Reg} Chip* constructed and validated here is available to the scientific community and has proven as a useful tool to study molecular mechanisms orchestrating T_{Reg} cells under physiologic and diseased conditions. In RCC and IBD patients, T_{Reg} cells were demonstrated to display not only numerical but also functional deficits that should be further dissected in order to unravel their remaining mysteries. Broadening our knowledge about these important immune regulators is essential for further development of new therapeutic approaches exploiting T_{Reg} cell potential in balancing peripheral tolerance.

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Weitere Publikationen

Franzke A, Geffers R, Hunger JK, **Pfoertner S**, Piao W, Ivanyi P, Grosse J, Probst-Kepper M, Ganser A, Buer J.: Identification of novel regulators in T-cell differentiation of aplastic anemia patients. *BMC Genomics*. 2006 Oct 19;7:263.

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